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Effects of a cocaine hydrolase engineered from human butyrylcholinesterase on metabolic profile of cocaine in rats

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Abstract

Accelerating cocaine metabolism through enzymatic hydrolysis at cocaine benzoyl ester is recognized as a promising therapeutic approach for cocaine abuse treatment. Our more recently designed A199S/F227A/S287G/A328W/Y332G mutant of human BChE, denoted as cocaine hydrolase-3 (CocH3), has a considerably improved catalytic efficiency against cocaine and has been proven active in blocking cocaine-induced toxicity and physiological effects. In the present study, we have further characterized the effects of CocH3 on the detailed metabolic profile of cocaine in rats administrated intravenously (IV) with 5 mg/kg cocaine, demonstrating that IV administration of 0.15 mg/kg CocH3 dramatically changed the metabolic profile of cocaine. Without CocH3 administration, the dominant cocaine-metabolizing pathway in rats was cocaine methyl ester hydrolysis to benzoylecgonine (BZE). With the CocH3 administration, the dominant cocaine-metabolizing pathway in rats became cocaine benzoyl ester hydrolysis to ecgonine methyl ester (EME), and the other two metabolic pathways (*i.e.* cocaine methyl ester hydrolysis to BZE and cocaine oxidation to norcocaine) became insignificant. The CocH3-catalyzed cocaine benzoyl ester hydrolysis to EME was so efficient such that the measured maximum blood cocaine concentration (~38 ng/ml) was significantly lower than the threshold blood cocaine concentration (~72 ng/ml) required to produce any measurable physiological effects.

Keywords

Enzyme; drug abuse; drug metabolism; metabolite; cocaine hydrolase

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Additional Information

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Introduction

Cocaine is known as the most reinforcing drug in stimulating the reward pathway of the brain and teaching the user to take it again [1–3]. Despite decades of effort, classical pharmacological approach to antagonizing the drug's neuropharmacological actions has not proven successful for cocaine, because it would be extremely difficult to antagonize cocaine's physiological effects without affecting normal functions of central nervous system (CNS) [4]. The inherent difficulties of antagonizing cocaine in the CNS led to the development of protein-based therapeutic agents that can tightly bind with cocaine and, thus, prevent cocaine from reaching the CNS or accelerate cocaine metabolism [4, 5]. Particularly, the pharmacokinetic approach with an efficient cocaine-metabolizing enzyme is recognized as the most promising treatment strategy for cocaine overdose and addiction [4, 6–8]. Unlike the stoichiometric binding of an antibody with drug, one enzyme molecule can degrade multiple drug molecules, and the catalytic efficiency of an enzyme is dependent on its catalytic rate constant (k_{cat}) and Michaelis-Menten constant (K_{M}) against the drug.

In humans, the principal metabolic enzyme of cocaine is butyrylcholinesterase (BChE) in the plasma, producing biologically inactive metabolites. However, the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of wild-type human BChE against (-)-cocaine (the naturally occurring enantiomer of cocaine) is too low ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$ and $K_{\text{M}} = 4.5 \text{ }\mu\text{M}$) [9] to be effective for (-)-cocaine metabolism. For convenience, below we will always refer cocaine to (-)-cocaine unless explicitly stated otherwise. In previous studies, we successfully designed and discovered human BChE mutants with at least 1,000-fold improved catalytic efficiency against cocaine compared to wild-type human BChE, and these BChE mutants are recognized as *true* cocaine hydrolases (CocHs) in literature [8, 10–13]. The first one of our designed CocHs, known as CocH1 (the A199S/S287G/A328W/Y332G mutant of human BChE) [10, 14], was fused with human serum albumin (HSA) to prolong the biological half-life [8], and the obtained HSA-fused CocH1 is also known as Albu-CocH, Albu-CocH1, AlbuBChE, or TV-1380 in literature [6–8, 15]. Clinical trials demonstrated that TV-1380 is safe and effective for use in animals and humans [6, 7], but its actual therapeutic value for cocaine addiction treatment is still limited by the moderate biological half-life (~8 hr in rats [8] or 43–77 hr in humans [6]). Generally speaking, the biological half-life of a therapeutic protein in humans is significantly longer than that in rats [8].

Notably, our more recently reported A199S/F227A/S287G/A328W/Y332G mutant of human BChE, known as CocH3 (with $k_{\text{cat}} = 5700 \text{ min}^{-1}$ and $K_{\text{M}} = 3.1 \text{ }\mu\text{M}$ against cocaine) [11, 16], is significantly more active against cocaine compared to CocH1. Hence, more recently reported animal studies were mainly focused on CocH3. It has been shown that CocH3 can effectively block cocaine-induced hyperactivity [16]. With pretreatment of 2 mg/kg CocH3 *via* intravenous (IV) injection, 25 mg/kg cocaine *via* intraperitoneal (IP) injection did not induce any notable hyperactivity in mice. In addition to the *in vivo* testing of CocH3 as a potential therapeutic enzyme for treatment of cocaine abuse, the gene therapy approach was also used to test CocH3 for its effectiveness and safety using viral gene transfer vectors encoding CocH3 [17–20]. It has been demonstrated that the gene transfer using a viral vector encoding CocH3 can yield plasma CocH3 levels that effectively detoxify

cocaine, block cocaine-induced physiological effects, and diminish long-term cocaine intake for lengthy periods without immune reactions or cholinergic dysfunction[17–20].

Despite of the extensive studies on CocH3, all of the animal studies on CocH3 reported so far have been focused on the effects of CocH3 on animal behaviors through accelerating cocaine benzoyl ester hydrolysis. It has also been known that cocaine is metabolized in the body *via* multiple metabolic pathways [10, 21, 22], as indicated in Figure 1. A remaining question is whether CocH3, an enzyme which can only accelerate cocaine benzoyl ester hydrolysis pathway, can indirectly affect the other metabolic pathways of cocaine. Further, if the answer to this question is “yes”, how will CocH3 affect the detailed metabolic profile of cocaine? The present study was designed to address these questions by using a sensitive LC-MS/MS method to determine blood concentrations of cocaine and its metabolites associated with three metabolic pathways in rats injected with cocaine after the rats are pretreated with CocH3 or saline. The data obtained under the same experimental conditions demonstrate that CocH3 indeed can dramatically change the detailed metabolic profile of cocaine in a favorable way.

Materials and Methods

Materials

Cocaine, norcocaine, benzoylecgonine (BZE), and ecgonine methyl ester (EME) were from National Institute of Drug Abuse (Bethesda, MD). Cocaine-D3, EME-D3, and BZE-D3, used as internal standards (IS), were purchased from Cerilliant (Round Rock, TX). Heparin, HPLC-grade methanol and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA). Ammonium hydroxide and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Mixed cation exchange model solid phase extraction cartridges (Oasis MCX 1 cc Vac Cartridge, 10 mg) were obtained from Waters (Milford, MA).

The purified CocH3 protein used in this study was prepared in our previous study [16]. CocH3 was purified by using a two-step approach, including ion exchange chromatography using Q sepharose fast flow anion exchange and affinity chromatography using procainamidesepharose [16]. The purified protein was dialyzed and stored at –80°C before the use.

Animal tests and sample collection

Male Sprague–Darley rats were ordered from Harlan (Indianapolis, IN), and housed initially as one or two rats per cage. All rats were allowed ad libitum access to food and water and maintained on a 12 h light/12 h dark cycle, with the lights on at 8:00 a.m. at a room temperature of 21–22 °C. Experiments were performed in a same colony room in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The animal protocol was approved by the IACUC (Institutional Animal Care and Use Committee) at the University of Kentucky. Rats (n=4 for each group) received IV injection of saline or 0.15 mg/kg CocH3 followed by cocaine administration (5 mg/kg, IV). Blood samples (50–75 µl) were collected into a heparin-treated capillary tube at 2, 5, 10, 15, 30, 60, 90, 120, 150, and 180 minutes after the

cocaine administration, and mixed with 100 μ l paraoxon solution (250 μ M paraoxon, 10 U/ml heparin) immediately. Blood samples were stored at -80°C until sample extraction.

Treatment of blood samples for mass spectrometry

The frozen blood samples were thawed on dry bath at 25°C for 15 min. Then, internal standard solution (100 nM for each internal standard) with a volume equal to that of the whole blood was mixed with each blood sample. The mixture was vortexed for 1 min and then centrifuged for 15 min at 13,000 rpm; and the supernatant was transferred to another clean centrifugal tube. After being added 500 μ l 4% formic acid, the sample was vortexed for 1 min and centrifuged at 13,000 for 15 min; and then the supernatant was submitted to solid-phase extraction. Oasis MCX 1 cc Vac Cartridge was preconditioned with 1 ml methanol followed by 1 ml water. Loaded cartridge was washed twice with 1 ml methanol, and the contents were eluted twice with 500 μ l methanol/water solution (95:5, v/v, with 7.5% ammonium hydroxide). Eluate was evaporated to dryness at 25°C using a vacuum concentrator, reconstituted in 74 μ l mobile phase A/B (95:5, v/v) (see below for the mobile phase compositions), and centrifuged at 13,000 rpm for 15 min. Supernatant was transferred to a vial and stored refrigerated until analysis by LC-Q-TOF (see below).

Preparation of stock, calibration standards and quality control samples

Combined stock solution was prepared by mixing solutions of cocaine and its metabolites with those of the corresponding deuterium-labeled internal standards. Specially, cocaine-D3 was used as an internal standard for both cocaine and norcocaine. The final concentration is 10 μ M for each analyte, and 0.1 μ M for each internal standard. Combined standard solutions, prepared by diluting the combined stock solution using internal standard solution (0.1 μ M for each internal standard), with the various concentrations for each analyte and 0.1 μ M for each corresponding internal standard. Quality control samples were prepared in the same manner. Calibration standards were prepared by adding 74 μ l different concentrations of combined standard solutions into 174 μ l blood mixture (74 μ l whole blood + 100 μ l paraoxon solution) from untreated Sprague-Dawley rats. The same method as described above was used to extract cocaine, EME, BZE, norcocaine, and all internal standards.

Liquid chromatographic and mass spectrometric conditions

A Shimadzu HPLC system, consisting of a DGU-20A/3R degasser, LC-20AD binary pumps, CBM-20A controller, and SIL-20A/HT auto sampler, was used in this study (Shimadzu, Kyoto, Japan). The mass spectrometer utilized for this work was an AB SCIEX tripleTOF™ 5600 (AB SCIEX, Redwood City, CA).

The chromatographic analysis was carried out on an Atlantis T3 (100Å, 3 μ m, 2.1 mm X 150 mm I.D) column (Waters, Milford, MA). Mobile phase A consisted of 2 mM ammonium formate, pH 3.0 and mobile phase B consisted of mobile phase A: acetonitrile (10:90, v/v). The flow rate was set at 0.2 ml/min. A 5 μ l injection of each sample was loaded on to the column. The gradient program was as follow: 5% B at 0 min, hold 5% B for 3 min, then B increased to 20% at 4 min, 40% B at 14 min, 90% B at 16 min, hold 90% B for 4 min, 5% B at 21 min, and reequilibrate at 5% B for 3 min[23]. The total run time was 24 min, and the column temperature was maintained at room temperature ($\sim 21^{\circ}\text{C}$). The auto

sampler temperature was maintained at 15°C, and the auto sampler injection needle was washed with 200 µl mobile phase A: mobile phase B (95:5, v/v) after each sample injection.

The mass spectrometer was run in positive ion and high sensitivity mode under the following conditions and settings: positive ions were generated in the source using liquid nitrogen. Ion source gas 1 (GS1) and 2 (GS2) were set at 25 and 35 respectively, and curtain gas (CUR) was set at 40. Source gas temperature was set at 500°C. Ion spray voltage floating (ISVF) was 3000 V. Compound-specific ionization parameters, ion transition, declustering potential (DP), collision energies (CE), collision energy spread (CES), ion release delay (IRD), and ion release width (IRW) were optimized and summarized in Table 1. Analyst® TF 1.7 software (AB SCIEX, Redwood City, CA) was used for instrument control and data acquisition. MultiQuant™ 3.0 software (AB SCIEX, Redwood City, CA) was used for quantitative analysis.

Results and Discussion

As well known, cocaine is metabolized in the body *via* three different types of metabolic pathways [10, 21, 22]: BChE- and carboxylesterase-2 (CE2)-catalyzed cocaine hydrolysis at the benzoyl ester to ecgonine methyl ester (EME) and benzoic acid; carboxylesterase-1 (CE1)-catalyzed cocaine hydrolysis at the methyl ester group to benzoylecgonine (BZE) and methanol; liver microsomal cytochrome P450 3A4 (CYP3A4)-catalyzed cocaine oxidation to norcocaine. In order to examine the effects of Coch3 on the metabolic profile of cocaine, we determined the blood concentrations of cocaine and its metabolites including EME, BZE, and norcocaine. The data obtained are depicted in Figure 2 (the standard curves) and Figure 3 (time-dependent concentrations of cocaine and its metabolites).

Under the experimental conditions designed for this study, our LC-MS/MS method can reasonably detect the norcocaine concentration as low as ~1 ng/ml, and the cocaine, EME, and BZE concentrations as low as ~5 ng/ml in the blood samples. It should also be pointed out that the actual errors of the experimental measurements are also dependent on many other factors, such as the time after the cocaine injection and the absolute concentration of each compound at the time. For a given compound at the first time point (2 min), a minor error of a few seconds in the time could lead to a huge deviation in the measured concentration due to the rapid distribution/elimination/reactions of the compound within the first few minutes. Particularly when the concentration of a compound (*i.e.* cocaine with saline or EME with Coch3) at 2 min is very high, the absolute error of the measured concentration is expected to be much higher. So, the concentrations determined at 2 min are expected to be most inaccurate.

To better understand the experimental data in Figure 3, one should know the relationship between the plasma concentration and stimulant effects of cocaine [24]. Volkow *et al.* demonstrated that, for humans, “at least 47% of the transporters had to be blocked for subjects to perceive cocaine’s effects” and that 0.1 mg/kg cocaine (IV) producing ~97 ng/ml cocaine in plasma (the peak concentration) blocked 47% of the transporters [25]. In comparison, 0.05 mg/kg cocaine (IV) blocked only 41% of the transporters and, thus, had no measurable effects [25]. According to their observation [25], the threshold concentration of

cocaine in plasma required to produce measurable physiological effects should be between 48 and 97 ng/ml or $\sim 72 \pm 25$ ng/ml. This means that cocaine with a plasma concentration below ~ 72 ng/ml is not expected to produce measurable physiological effects.

It has been known that, in rats, the endogenous BChE is very inefficient in metabolizing cocaine and, for this reason, cocaine is mainly metabolized by CE1 in rats to produce BZE and methanol [26]. The control curves (associated with saline) in Figure 3 reflect the overall effects of all metabolic and elimination pathways for cocaine and its metabolites. As seen in Figure 3, in the control rats, the average blood concentration of cocaine at the first time point (2 min) was ~ 1700 ng/ml (much higher than the aforementioned threshold blood concentration of ~ 72 ng/ml), while the average blood concentration of EME was ~ 6 ng/ml, the average blood concentration of BZE was ~ 52 ng/ml, and the average blood concentration of norcocaine was ~ 2.0 ng/ml. The dominant metabolite at 2 min was BZE produced *via* cocaine methyl ester hydrolysis. After 2 min, the average blood cocaine concentration gradually decreased to ~ 240 ng/ml at 60 min, ~ 97 ng/ml (still higher than the threshold plasma concentration of ~ 72 ng/ml) at 90 min, and ~ 37 ng/ml at 120 min, while the blood concentrations of its metabolites (EME, BZE, and norcocaine) gradually increased. The observed peak metabolite concentration was ~ 35 ng/ml for EME at 60 min, ~ 910 ng/ml for BZE at 60 min, and ~ 3.3 ng/ml for norcocaine at 30 min. These data show that the dominant cocaine-metabolizing pathway in the control rats is cocaine methyl ester hydrolysis to BZE. The other two metabolic pathways of cocaine are less significant, but their metabolites (*i.e.* EME and norcocaine) are all detectable using the LC-MS/MS method.

As shown in Figure 3, in the presence of CocH3, the average blood concentration of cocaine at 2 min was as low as ~ 38 ng/ml (lower than the threshold plasma concentration of ~ 72 ng/ml), while the average blood concentration of EME was as high as ~ 2700 ng/ml and the average blood concentration of BZE was ~ 20 ng/ml. Meanwhile, the blood norcocaine concentrations were all negligible (below 1 ng/ml) throughout the observation period (3 hours). Most of cocaine was hydrolyzed by CocH3 between the IV cocaine injection and the first blood sampling at 2 min after the injection. After 2 min, the blood concentrations of cocaine and EME gradually decreased, while the BZE concentration first gradually increased to the peak (~ 113 ng/ml) at 30 min and then gradually decreased to 33 ng/ml after 3 hours. These data indicate that IV administration of 0.15 mg/kg CocH3 dramatically changed the metabolic profile of cocaine in rats. In the presence of CocH3, the dominant cocaine-metabolizing pathway became cocaine benzoyl ester hydrolysis to EME. The CocH3-induced dramatic changes in both the cocaine and EME concentrations clearly indicate that cocaine was metabolized rapidly to EME in the presence of CocH3. Due to the acceleration of cocaine benzoyl ester hydrolysis, the other two metabolic pathways of cocaine became insignificant. So, with the enzyme therapy using CocH3 as a pretreatment for cocaine addiction, we should not need to worry too much about the unwanted formation of norcocaine (which is more toxic than cocaine itself [27]) and BZE associated with the other metabolic pathways of cocaine.

Conclusion

The *in vivo* data obtained in this study demonstrate that IV administration of 0.15 mg/kg Coch3 dramatically changed the metabolic profile of cocaine in rats. In the absence of Coch3, the dominant cocaine-metabolizing pathway in rats (injected with 5 mg/kg cocaine, IV) was cocaine methyl ester hydrolysis to BZE. The other two metabolic pathways (*i.e.* cocaine benzoyl ester hydrolysis to EME and cocaine oxidation to norcocaine) were less significant, but their metabolites EME and norcocaine were all detectable using the LC-MS/MS method. In the presence of Coch3, the dominant cocaine-metabolizing pathway in rats (given 5 mg/kg cocaine, IV) became cocaine benzoyl ester hydrolysis to EME, and the other two metabolic pathways (*i.e.* cocaine methyl ester hydrolysis to BZE and cocaine oxidation to norcocaine) became insignificant. The cocaine benzoyl ester hydrolysis to EME was so rapid such that the average cocaine concentration (~38 ng/ml) remained in the blood samples taken at the first time point (2 min) was significantly lower than the threshold plasma cocaine concentration (~72 ng/ml) required to produce any measurable physiological effects of cocaine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- Cocaine was metabolized mainly through methyl ester hydrolysis in untreated rats.
- CocH3 administration dramatically changed the metabolic profile of cocaine in rats.
- Dominant metabolic pathway became cocaine benzoyl ester hydrolysis by CocH3.
- With CocH3, the other metabolic pathways of cocaine became insignificant.

Figure 1. Metabolic reactions of cocaine catalyzed by BChE (or CocH), carboxylesterase-1 (CE1), and cytochrome P450 (CYP) 3A4.

Figure 2.

LC-MS/MS calibration curves for cocaine, EME, BZE, and norcocaine. All analyte concentrations are given in ng/ml. Calibration curves were established by calculating the ratios of the peak area for analyte to that for the internal standard and plotting the ratio as a function of the ratio of the analyte concentration to the internal standard. X-axis (Analyte Conc. / IS Conc.) refers to the ratio of the analyte concentration to the corresponding internal standard concentration (IS Conc. = 0.1 μ M for each internal standard compound). Y-axis (Analyte Area / IS Area) represents the ratio of the measured area for the analyte to that for the corresponding internal standard. For each compound (cocaine or a metabolite), data were fitted to a linear curve using the least-squares analysis with 1/x weighting.

Figure 3. Metabolic profiles of cocaine in the presence and absence of CocH3. Rats (n=4 for each group) were injected (IV) with saline or 0.15 mg/kg CocH3 1 min before the IV injection of 5 mg/kg cocaine.

Table 1

Optimized compound-specific source parameters for electrospray ionization

Drugs	Ion transition	DP (V)	CE (eV)	CES (V)	IRD	IRW
Cocaine-D3	307.2→185.1	50	30	3.0	45	17
Cocaine	304.1→182.1	100	30	3.0	45	17
Norcocaine	290.1→168.1	50	25	3.0	45	17
EME-D3	203.1→185.1	60	30	3.0	45	17
EME	200.1→182.1	80	31	3.0	45	17
BZE-D3	293.2→171.1	60	30	3.0	45	17
BZE	290.1→168.1	60	28	3.0	45	17