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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.

1. 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 to produce biologically inactive metabolites. However, the catalytic efficiency (k_{cat}/K_M) of wild-type human BChE against (−)-cocaine (the naturally occurring enantiomer of cocaine) is too low (k_{cat} = 4.1 min^{-1} and K_M = 4.5 μM) [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 1000-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],

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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 h in rats [8] or 43–77 h 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_{cat} = 5700 min^{-1} and K_M = 3.1 μM 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 Fig. 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 when 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.

2. Materials and methods

2.1. 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).

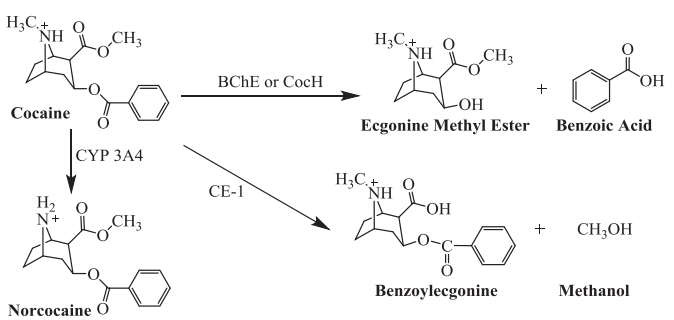

Fig. 1. Metabolic reactions of cocaine catalyzed by BChE (or CocH), carboxylesterase-1 (CE1), and cytochrome P450 (CYP) 3A4.

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 procainamide-sepharose [16]. The purified protein was dialyzed and stored at −80 °C before the use.

2.2. 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 min after the cocaine administration, and mixed with 100 μl paraoxon solution (250 μM paraoxon, 10 U/ml heparin) immediately. Blood samples were stored at −80 °C until sample extraction.

2.3. 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 μl 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 μl methanol/water solution (95:5, v/v, with 7.5% ammonium hydroxide). Eluate was evaporated to dryness at 25 °C using a vaccum concentrator, reconstituted in 74 μl 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).

2.4. 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 μM for each analyte, and 0.1 μM for each

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