Preparation and *in vivo* characterization of a cocaine hydrolase engineered from human butyrylcholinesterase for metabolizing cocaine

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Cocaine is a widely abused drug without an FDA (Food and Drug Administration)-approved medication. It has been recognized that an ideal anti-cocaine medication would accelerate cocaine metabolism producing biologically inactive metabolites via a route similar to the primary cocaine-metabolizing pathway, i.e. human BChE (butyrylcholinesterase)-catalysed hydrolysis. However, the native human BChE has a low catalytic activity against cocaine. We recently designed and discovered a BChE mutant (A199S/F227A/S287G/A328W/Y332G) with a high catalytic activity ($k_{cat} = 5700 \text{ min}^{-1}$, $K_m = 3.1 \mu\text{M}$) specifically for cocaine, and the mutant was proven effective in protecting mice from acute cocaine toxicity of a lethal dose of cocaine (180 mg/kg of body weight, LD$_{100}$). Further characterization in animal models requires establishment of a high-efficiency stable cell line for the BChE mutant production at a relatively larger scale. It has been extremely challenging to develop a high-efficiency stable cell line expressing BChE or its mutant. In the present study, we successfully developed a stable cell line efficiently expressing the BChE mutant by using a lentivirus-based repeated-transduction method. The scaled-up protein production enabled us to determine for the first time the *in vivo* catalytic activity and the biological half-life of this high-activity mutant of human BChE in accelerating cocaine clearance. In particular, it has been demonstrated that the BChE mutant (administered to mice 1 min prior to cocaine) can quickly metabolize cocaine and completely eliminate cocaine-induced hyperactivity in rodents, implying that the BChE mutant may be developed as a promising therapeutic agent for cocaine abuse treatment.

Key words: butyrylcholinesterase, drug abuse, protein drug, protein production, stable cell line.

INTRODUCTION

Cocaine is one of the most addictive drugs. Despite decades of efforts in developing effective anti-cocaine therapies, there is still no FDA (Food and Drug Administration)-approved pharmacological treatment specific for cocaine overdose or addiction. Enzyme therapy has been recognized as an ideal approach to anti-cocaine medication for accelerating cocaine metabolism, producing biologically inactive metabolites via a route similar to the primary cocaine-metabolizing pathway, i.e. hydrolysis catalysed by human BChE (butyrylcholinesterase) [1–3]. Unfortunately, the native BChE has a low catalytic activity against naturally occurring biologically active (–)-cocaine ($k_{cat} = 4.1 \text{ min}^{-1}$, $K_m = 4.5 \mu\text{M}$). We developed unique computational strategies and protocols based on virtual screening of rate-determining transition states of the enzymatic reaction to design enzyme mutants with an improved catalytic activity [4–9]. The computational design was followed by *in vitro* experiments, including site-directed mutagenesis, protein expression and fast enzyme activity screening. The integrated computational experimental studies led to the discovery of a set of high-activity BChE mutants known as cocaine hydrolases [4–10]. One of our designed and discovered mutants, A199S/S287G/A328W/Y332G [4,5] has been validated by an independent research group [11–14] and recognized as “a true cocaine hydrolase with a catalytic efficiency that is 1000-fold greater than wild-type BChE” [14]. Our more recently reported A199S/F227A/S287G/A328W/Y332G mutant (denoted as CocH3 for convenience) demonstrated a ∼2000-fold improved catalytic activity ($k_{cat} = 5700 \text{ min}^{-1}$, $K_m = 3.1 \mu\text{M}$) [6] against (–)-cocaine compared with the wild-type BChE. In the *in vivo* potency test, pretreatment with 10 μg of CocH3 fully protected the mice from the acute toxicity of 180 mg of cocaine/kg of body weight (LD$_{100}$). These high-activity mutants of human BChE have been recognized as promising candidates of therapeutic enzymes for anti-cocaine medication [1–3,15]. In the initial screening of those BChE mutants, the genes of the mutants were transiently expressed for *in vitro* and *in vivo* examination. Further characterization of this enzyme in animal models requires a stable expression cell line efficiently producing the enzyme in large quantities.

Traditionally, generation of a stable cell line for therapeutic protein production begins with construction of an expression vector. A suitable vector (plasmid or virus) usually carries the gene for the target protein and a metabolic selectable marker or an antibiotics selectable marker for the cell line of choice. After transfection with the vector, the cells are then grown under the selection pressure and screened for the expression level of the target protein. The high-productivity clones are selected and amplified for scaled-up production. For this, the integration position and plasmid copy number affect the productivity of the cells. One of the latest developed strategies is site-specific integration. Since only 0.1–1 % of the genome region are actively transcribed, integration of the target gene into specific loci would ensure high and stable productivity [16–18]. Several recombinases, such as Cre and Flp, were utilized for this purpose because of their capability to identify specific sequences and to mediate the integration of the foreign gene for the therapeutic protein(s) [16,17]. In all of the approaches mentioned above, the selection and screening for highly productive single clones is a time-consuming process which may take months.

Abbreviations used: BChE, butyrylcholinesterase; CHO, Chinese-hamster ovary; DHFR, dihydrofolate reductase; FDA, Food and Drug Administration; HEK, human embryonic kidney; QFF, Q Sepharose® Fast Flow; TFA, trifluoroacetic acid.

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Currently, CHO (Chinese-hamster ovary) cells are used for production of approximately 70% of therapeutic proteins [18]. They have been proven to be safe and able to provide efficient proper post-translational modification of proteins. However, CHO cells are also difficult to transfact and unstable for the foreign genes. So the commonly used forms of CHO cells are genetically modified, such as the DHFR (dihydrofolate reductase)-deficient CHO cell lines DUXB11 and DG44 [17,18]. The expression vector will carry both the gene encoding the lacking enzyme (such as DHFR) with an impaired promoter and the gene for expression of the target protein. After the transfection and metabolic selection, the target gene could be stabilized and amplified by the addition of an increasing dose of an inhibitor for the lacked enzyme (such as DHFR). Recombinase-aided site-specific integration is also applied alone or in combination with the gene amplification system to achieve establishment of highly efficient stable cell lines [16,17].

There have been many efforts in developing high-efficiency stable cell lines to produce BChE at a relatively larger scale. A traditional transfection-selection method has been used to generate stable CHO cell lines that yield approximately 3–5 mg/l pure BChE or mutant [19]. Insect cells, which can produce monoclonal antibody with a yield of 52 mg/l per day in batch culture [20] are generally considered as a highly efficient expression system to produce recombinant proteins. However, when expressing a truncated human BChE mutant, insect cells can only achieve a production level of 4 mg/l [21]. It is difficult to generate cell lines that can stably express BChE or its mutant at a high level. Alternatively, transgenic animals/plants strategies [22,23] have succeeded in yielding recombinant BChE and mutants at high production levels. But the proteins produced have other problems. For example, recombinant BChE produced in transgenic goats has a short halflife [22]. In addition, it requires a long period of time to generate the transgenic animals or plants. It is challenging to develop an effective method for scaled-up production of BChE or its mutant.

In the present paper we report the use of a lentivirus-based repeated transduction approach which transduces the prepared lentivirus into CHO cells repeatedly for stable cell line generation. This approach has led us to successfully generate a stable cell line which can efficiently produce CocH3 (the high-activity mutant of human BChE). In addition, a stable HEK (human embryonic kidney)-293F cell line was also developed for comparison. The prepared CocH3 protein was characterized in vivo for its pharmacokinetic properties and effectiveness in accelerating cocaine metabolism and eliminating cocaine-induced hyperactivity.

**MATERIALS AND METHODS**

**Preparation of lentivirus encoding the gene of the BChE mutant**

The BChE mutant (CocH3) cDNA in lentivirus plasmid was constructed into the pCSC-SP-PW vector at ApaI and XhoI sites. The A1995/F227A/S287G/A328W/Y332G mutations were generated on cDNA of full-length human BChE (accession number P06276 in the Swiss Protein Database) on pRC/CMV-BChE by using site-directed mutagenesis. The CocH3 gene was amplified by PCR with pRC/CMV-CocH3 as a template. The forward primer was 5′-GGAGGGCCCAAGGTGCACGGCCACAGTAGT-3′ (ApaI), and the reverse primer was 5′-CGCTGAGGTAGAAGACCCACACACTTCTC-3′ (XhoI). Then the CocH3 cDNA was ligated with the pCSC-SP-PW fragment that was double-digested by ApaI and XhoI. The construct sequence was confirmed by DNA sequencing. To package the lentivirus particles carrying the gene of CocH3, HEK-293FT cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium)-10% FBS (Life Technologies) with 250 ng/ml G418 (Life Technologies). Cells were transfected at approximately 70% confluence by lentivirus plasmid encoding CocH3 (CocH3/pCSC-SP-PW) along with three other packaging plasmids, pMDL-pg.RRE, pRSV.rev and pSVG, at a mass ratio of 10:6.5:2.5:3.5. Transfection was achieved by lipofection. In brief, for a 10-cm diameter dish of cells, total DNAs of approximately 22.5 μg were mixed first, and then diluted in 1.5 ml of Opti-MEM® I Reduced Serum Medium (Invitrogen) without serum. An aliquot (60 μl) of Lipofetamine™ 2000 (Invitrogen) was then mixed with 1.5 ml of Opti-MEM® I Reduced Serum Medium and incubated at room temperature (25°C) for 5 min. The diluted DNAs and Lipofetamine™ 2000 were mixed and incubated at room temperature for 20 min before being added dropwise on to the cell culture. The cells were cultured at 5% CO2 at 37°C. Culture medium was changed 12–16 h after transfection. The medium was collected three times at a 24-h-interval beginning 24 h after the post-transfection change of medium. The medium was filtered through a 0.45-μm cellulose acetate filter and spun in a Beckman SW28 rotor at 19400 rev./min for 2 h at 4°C to pellet the virus particles. Lentivirus was then suspended in Hank’s balanced salt solution and aliquoted to be stored at –80°C. The physical concentration of lentivirus was determined by using the QuickTiter™ lentivirus rapid quantitation kit (Cell Biolabs).

**Generation of the stable cell line by lentivirus infection**

Scale-up preparation of enzyme was first achieved by infecting CHO-S cells with lentivirus followed by resuspending attached CHO-S cells in protein-free suspension culture in Gibco® FreeStyle™ CHO expression medium (Life Technologies) with 8 mM L-glutamine. The day before infection, the cells were loaded at 1×10⁶ cells/ml in a 12-well plate and cultured steadily in FreeStyle™ CHO expression medium with 8 mM L-glutamine and 1% FBS. Cells began to attach to the plate quickly in the presence of FBS after the change in culture conditions. Lentivirus was then added to infect the cells for 1 day. The cross-linker Polybrene® (Santa Cruz Biotechnology) was also added to the cell culture to increase the infection efficiency by neutralizing the charge repulsion between the virus and cell culture. The infection was stopped by change of medium. The infected cells were allowed to recover from the infection for 1–2 days (or more days, depending on the status of the cells), then were suspended using 0.05% trypsin-EDTA and split into two. One half was cultured in a six-well plate, and then transferred to a 10-cm diameter plate in 1% FBS FreeStyle™ CHO expression medium. Then the cells were changed back to suspension culture, and the culture volume was increased from a six-well plate to 125-ml shake flask for yield determination. The other half was seeded in a 12-well plate for the next round of infection. After each infection, the yield was determined for a 9-day fermentation of 30 ml of cell culture. The pool with the highest yield was selected and amplified for scale-up production of the enzyme. Culture medium was changed every 2–3 days, and collected for storage at 4°C. The scalability and stability of the production was also evaluated during the process. The stable cell line in HEK-293F was also generated by using the same method. For the difference, the HEK-293F cells were cultured in Gibco® FreeStyle™ 293 medium (Life Technologies), with the addition of 1 μM poly-L-proline (Sigma–Aldrich) to facilitate the formation of the CocH3 tetramer.

To compare the productivity of the pool with that of single clones, the cells from the pool were seeded at 2–10 cells/well.
in 96-well plates in FreeStyle™ CHO expression medium with 1% FBS to select the single clones. The cells were cultured for another 14–21 days without changing the medium and shaking until single clones clearly appeared. Single clone cell lines were chosen to culture in 48-well plates, then 12-well plates and six-well plates in 1% FBS FreeStyle™ CHO expression medium. High-expression single clones were selected by determining the enzyme activity in the medium. Selected cell-line cells were then changed to suspension culture and the culture volume was changed from a six-well plate to a 125-ml flask. The yields of single clones were determined for a 9-day fermentation of 30 ml of cell culture.

Enzyme purification

Scale-up purification of the enzyme in the medium was achieved by a two-step purification using ion-exchange chromatography followed by affinity chromatography. In brief, the crude medium was diluted with the same volume of 20 mM Tris/HCl, pH 7.4. Equilibrated QFF (Q Sepharose® Fast Flow) anion exchanger was added to diluted medium in 1% of its volume and incubated at 4°C with occasional stirring for 1 h. More than 95% enzyme activity was found to bind to the resin after the incubation. The suspension was then packed in a column and the medium was allowed to flow through rapidly with the aid of suction at 50–75 ml/min. The QFF resin was repacked again in a washing buffer after the entire medium was excluded. After washing the column with 20 mM Tris/HCl, pH 7.0, overnight at 4°C, the enzyme was eluted using 20 mM Tris/HCl, pH 7.0, and 0.3 M NaCl. The eluate was desalted to 20 mM Tris/HCl, pH 7.0, and when an equal amount of 4°C with occasional stirring for 15 min. The serum was tested for enzyme activity using a radiometric method as described in our previous studies [6,7,10]. The data for elimination of the enzyme from the circulation were fitted to a double-exponential equation which was described previously by Kronman et al. [25].

Non-denaturing gel electrophoresis

Activity-stained non-denaturing polyacrylamide gels were utilized to estimate the relative amount of tetramers, dimers and monomers. Polyacrylamide stacking gel (4%) and 8% separating gel were prepared in a Bio-Rad gel apparatus. Electrophoresis was at 8 mA constant current for 6 h at 4°C. The gel was stained for BChE activity against 2 mM butyrylthiocholine iodide by using the method described by Karnovsky and Roots [24].

Subjects for in vivo studies

Sprague–Dawley (male or female) rats (200–250 g) and male CD-1 mice (27–30 g) were ordered from Harlan. The rats were housed initially as two to four rats per cage. The mice were housed in groups of two to five mice per cage. All of the animals were allowed ad libitum access to food and water and were maintained on a 12-h light and dark cycle with lights on at 08:00 in a room kept at a temperature of 21–22°C. Each animal was only used once. Experiments were performed in the 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 experimental protocols were approved by the IACUC (Institutional Animal Care and Use Committee) at the University of Kentucky.

Determination of in vivo half-life in rats

Rats were injected intravenously (via the tail vein) with 0.15 mg/kg of body weight of the purified enzyme expressed in the stable CHO-S or HEK-293F cells. The saphenous veins were punctured with a needle. Approximately 50–75 μl of blood was collected into a capillary tube at various time points after the enzyme injection. The blood samples were centrifuged at 5000 g for 15 min. The serum was tested for enzyme activity using a radiometric method as described in our previous studies [6,7,10].

Characterization of cocaine clearance accelerated by CocH3

The general anaesthetic isoflurane was utilized with a nose cone during the course of study. Four rats were injected with saline through the tail vein 1 min before intravenous injection of 5 mg of cocaine/kg of body weight, and another four rats were injected with 0.1 mg of CocH3/kg of body weight followed by an intravenous injection of the same dose of cocaine. Approximately 50–75 μl of blood from saphenous veins was collected into capillary tubes and immediately diluted in 100 μl of 250 μM paraaxon 2, 5, 10, 15, 30 and 60 min after the intravenous injection of cocaine. Paraaxon is an irreversible BChE inhibitor that can stop the enzymatic hydrolysis of cocaine between sampling and analysis. The diluted blood samples were stored at −70°C and assayed using HPLC.

Benzoic acid is the product of cocaine hydrolysis by the enzyme. Standard cocaine and benzoic acid were purchased from Sigma–Aldrich. Cocaine and benzoic acid concentrations in the blood samples were assayed by the following method. The frozen whole blood samples were thawed on ice for 3 h. Then 150 μl of mobile phase [74% acetonitrile and 0.26% TFA (trifluoroacetic acid)] was mixed with each sample. Then 50 μl of 7% HClO₄ was added to break the blood cell membrane. The mixture was vortex mixed for 1 min and then centrifuged at 25000 g for 15 min, and the supernatant was transferred to an autosampler vial of which 200 μl was injected into the chromatographic system.

Chromatography was performed using a Waters 1525 binary HPLC pump, a Waters 2487 dual λ absorbance detector, a Waters 2475 multi λ fluorescence detector, and a Waters 717 plus autosampler. The mobile phase was 74% acetonitrile and 0.26% TFA. The flow rate was 1 ml/min. The eluent was monitored at 230 nm for absorbance of benzoic acid and at 465 nm for fluorescence of cocaine when exciting at 383 nm. The cocaine peaks appeared at 10.5 min, and the benzoic acid peaks occurred at 14.5 min. The quantification of cocaine and benzoic acid was performed by comparing the corresponding HPLC peak areas with those of authentic standards.

Locomotor activity

The mice were acclimatized to live individually in the cages for at least 1 h. Mice were injected with cocaine alone, saline alone or the BChE mutant (CocH3) 1 min before 25 or 90 mg of intraperitoneal cocaine/kg of body weight. The CocH3 doses were varied from 1.5 to 5 mg/kg of body weight (intravenous). The mice were returned to cages after the intraperitoneal injection of cocaine, and their locomotor activities were recorded for 10 min. The distances travelled within the time were evaluated.
RESULTS AND DISCUSSION

Productivity of the stable cell line

We first wanted to explore an efficient method to generate a high-productivity cell line stably expressing a BChE mutant. The BChE mutant cDNA in lentivirus plasmid was constructed into the pCSC-SP-PW vector. The lentivirus was packed in HEK-293FT cells by transfection of pCSC-SP-PW-BChE along with three other packaging plasmids and then purified by centrifugation. The CHO-S cells were loaded on to 12-well plates and transduced by adding purified lentivirus repeatedly. After each infection, the yield of transduced cells was determined in a 9-day fermentation of 30 ml of cell culture. The use of the lentivirus-based repeated transduction method resulted in a stable CHO-S cell line efficiently expressing CocH3 (BChE mutant). The production level is related to the number of times that transduction was performed, as seen in Figure 1. The productivity of the cell pool was increased after each additional round of the transduction–recovery cycle until after the seventh time (Figure 1B). The data suggested that, during the process, more copies of the target gene were integrated into the genome for enhanced target protein expression. After the seventh time, further transduction no longer increased the yield of the 9-day fermentation. In fact, after the seventh transduction, further transduction decreased the yield gradually. The decrease in the yield might be due to the possibility that the hot-spots of chromosomal loci were already saturated by the foreign genes after the seventh transduction and the random insertion into other regions of genome might harm the health of the cells. The amount of enzyme produced was proportional to the incubation time during the first few days when the nutrient was sufficient (Figure 1A). Then, the cells started to die due to lack of nutrients and accumulation of toxins such as lactate. The production rate gradually slowed down until the production level hit a plateau when all of the cells died.

We also selected single clones from the pool and determined the yields associated with the single clones (Figure 2). The single clones did not display significantly higher or lower yields in the 9-day fermentation test compared with the pool, suggesting that the cells were transduced rather homogenously such that almost all of the cells had a similar productivity. On the basis of this observation, in future work we may simply skip the step of the selection of single clones so as to save a lot of time during the development of stable cell lines for other BChE mutants.

Since CHO cells are known to be difficult in sustaining the expression level of foreign genes, we also evaluated the scalability and stability of the established CocH3-expressing cell line. For the stability test, the cells were seeded at $6 \times 10^5$ cells/ml after each passage and cultured for 3 days, then one-eighth of them was passaged while the rest were cultured continuously for 6 days to determine the yield of the 9-day fermentation. As seen in Figure 3, after several passages, the cells did not significantly decrease their productivity. In the scalability test, the yield rate did not significantly change by increasing the volume of the cell culture.
from millilitres to litres. So the cells were sustainable and scalable for protein production.

Figure 4 depicts a non-denaturing gel (8%) stained for the BChE activity of the CocH3 materials expressed in HEK-293F cells (with 1 μM poly-L-proline in the medium) and CHO-S cells. According to the data in Figure 4, CocH3 materials expressed in the HEK-293 cells (with 1 μM poly-L-proline in the medium) and the CHO-S cells all predominantly existed as a tetramer. For CocH3 expressed in the HEK-293F cells (with 1 μM poly-L-proline in the medium), there was no band observed for the monomer or dimer. For CocH3 expressed in the CHO-S cells, a very weak (wide) band was noted for the monomer (with a negligible amount), but no dimer was noted. In comparison, weak (wide) bands were noted for both the monomer and dimer in CocH3 expressed in the transiently transfected CHO-S cells (with 1 μM poly-L-proline in the medium).

Concerning the catalytic activity of the BChE mutant, according to our previously reported in vitro assay [6], we determined that $k_{cat} = 5700$ min$^{-1}$ and $K_m = 3.1$ μM for CocH3 expressed in HEK-293F cells. The same in vitro assay (the sensitive radiometric assay) was employed to determine the enzymatic activity of CocH3 expressed in the stable CHO-S cells, showing no significant change in the catalytic activity (results not shown).

Pharmacokinetic profiles of CocH3

The CocH3 protein material expressed in the stable CHO-S cells was tested for its pharmacokinetic profile in rats. Rats ($n = 5$) were administered intravenously (via tail vein) with 0.15 mg/kg of body weight of the purified enzyme. The blood was sampled at 2, 15 and 30 min, and 1, 2, 3, 5, 8, 12, 24 and 48 h after the enzyme injection. For comparison, the protein material expressed in the stable HEK-293F cells was also tested in rats. Figure 5 shows the time courses of the active enzyme concentrations after the intravenous injection of the enzyme materials. The measured time-dependent concentrations of the active enzyme were fitted to a well-known double exponential equation $(E(t) = A e^{-k1t} + B e^{-k2t})$ which accounts for both the enzyme distribution process (the fast phase, associated with $k_1$) and elimination process (the slow phase, associated with $k_2$). The half-life associated with the enzyme elimination rate constant $k_2$ is called the biological half-life $(t_{1/2})$.

The CHO cells expressed CocH3 at a biological half-life of 7.3 h, which is significantly longer than that (2.8 h) of the same enzyme expressed in the HEK-293F cells, in rats. The native human BChE (purified from human serum) has a half-life of 7–12 days in humans and 24 h in rats [26]. Recombinant BChE has a much shorter biological half-life [27]. It should be noted that multiple factors could affect the biological half-life of a protein, including oligomerization and glycosylation. As the non-denaturing gel staining (Figure 4) revealed that the CocH3 proteins expressed in the HEK-293F and CHO-S cells were predominantly a tetramer, there was no significant difference in oligomerization between the two protein forms. Thus the difference in the biological half-life is mainly due to the difference in post-translational modification, particularly glycosylation. The CHO cell CocH3 expression may have glycosylation closer to the native enzyme compared with that for the CocH3 expressed in HEK-293F cells.

Generally speaking, it is desirable to have a long in vivo half-life for the potential therapeutic protein in cocaine addiction treatment. The longer the enzyme can stay in the body, the longer it can protect the subjects, and the lower dosing and/or lower frequency the therapeutic protein would be needed for administration to the patients. It also lowered the potential risk of unexpected adverse effects, and increased the chances of full protection of patients against cocaine effects. On the basis of the results depicted in Figure 5, future CocH3 production should use the stable CHO-S cell line.

Cocaine clearance is accelerated by CocH3

In order to examine the in vivo potency of CocH3 for metabolizing cocaine, we characterized the pharmacokinetic profiles of cocaine clearance with and without the presence of CocH3 in rats by using a chromatographic assay. The data are depicted in Figure 6. The rats ($n = 4$) were injected with saline or 0.1 mg of CocH3/kg of body weight, followed by intravenous injection of 5 mg of cocaine/kg of body weight. The blood was sampled at 2, 5, 10, 15, 30 and 60 min after the cocaine injection. The blood samples were analysed for the concentrations of cocaine and benzoic acid by using an HPLC method. CocH3 can hydrolyse cocaine to produce benzoic acid and ecgonine methyl ester, and greatly accelerate the clearance of cocaine from the body. It has been known that the endogenous BChE in rats is very inefficient in metabolizing cocaine and, for this reason, cocaine was mainly metabolized by carboxylesterase in the blood to produce...
concentration of cocaine at ∼ relatively smaller distribution volume compared with cocaine. Whereas benzoic acid primarily exists as the benzoate ion under physiological conditions, it can readily cross cell membranes under physiological conditions, as is well known, cocaine is an amine drug which rapidly to benzoic acid in the presence of CocH3. The concept of a possible enzyme therapy for cocaine abuse treatment is based on a hypothesis that the effects of cocaine are dependent on how intensely and how quickly cocaine gets to the brain, and that the therapeutic enzyme can alter the pharmacokinetics of cocaine in a favourable manner by rapidly metabolizing benzoylecgonine and methanol in rats [26]. The control curves in Figure 6 reflect the overall effects of all of the cocaine elimination pathways. As seen in Figure 6, in the control rats, the average concentration of cocaine at the first time point (2 min) was 7.4 μM, whereas the average concentration of benzoic acid (metabolite) was 0.5 μM. In the presence of CocH3, the average concentration of cocaine at ∼ 2 min in the blood sample was below the detectable level (Figure 6A), whereas the average concentration of benzoic acid at the first time point (2 min) was 12.2 μM (Figure 6B). Most of the cocaine was hydrolysed by CocH3 between the intravenous cocaine injection and the first blood sampling at 2 min after the injection. The CocH3-caused dramatic changes in both the cocaine and benzoic acid concentrations clearly indicated that cocaine was metabolized rapidly to benzoic acid in the presence of CocH3.

It should be mentioned that the total plasma concentration of cocaine and benzoic acid (12.2 μM) in the presence of CocH3 (when the benzoic acid concentration was higher) was higher than that (7.4 μM) in the absence of CocH3 (when the cocaine concentration was higher). This observation might be associated with the potentially different distribution volumes of cocaine and benzoic acid in the body. As is well known, cocaine is an amine drug which can readily cross cell membranes under physiological conditions, whereas benzoic acid primarily exists as the benzoate ion under physiological conditions. Thus benzoic acid is expected to have a relatively smaller distribution volume compared with cocaine.

Figure 6 Cocaine clearance is accelerated by CocH3 expressed in CHO-S cells

Benzoic acid (B) is the product of BChE- or CocH3-catalysed hydrolysis of cocaine (A). Saline or 0.1 mg of CocH3/kg of body weight was injected intravenously into rats (n = 4) 1 min before the intravenous injection of 5 mg of cocaine/kg of body weight.

Figure 7 Effects of the exogenous enzyme (CocH3 expressed in stable HEK-293F cells) on cocaine-induced hyperactivity of mice

Saline or enzyme was injected intravenously through tail veins of mice 1 min before intraperitoneal injection of saline or cocaine. Five dose conditions were tested using five groups of mice, and each group had six mice (n = 6). Group a were treated with intravenous saline and intraperitoneal saline; group b were treated with intravenous saline and intraperitoneal 25 mg of cocaine/kg of body weight; group c were treated with intravenous 1.5 mg of CocH3/kg of body weight and intraperitoneal 25 mg of cocaine/kg of body weight; group d were treated with intravenous 2 mg of CocH3/kg of body weight and intraperitoneal 25 mg of cocaine/kg of body weight; and group e were treated with intravenous 5 mg of cocaine/kg of body weight and intraperitoneal 90 mg of cocaine/kg of body weight.

Effects of CocH3 on the hyperactivity induced by cocaine

Development of stable cell lines for scaled-up protein production enabled us to characterize the potency of the enzyme in elimination of the physiological effect of cocaine. In the present study, mice (n = 6) were injected with cocaine alone, saline alone or the BChE mutant (CocH3) 1 min before 25 or 90 mg of intraperitoneal cocaine/kg of body weight. The mice were returned to the cages and recorded for their locomotor activity for the first 10 min after the cocaine injection. The first cocaine dose used for our locomotor activity tests in mice was 25 mg of cocaine/kg of body weight (intraperitoneally). As seen in Figure 7, without pretreatment of the enzyme, 25 mg of cocaine/kg of body weight (intraperitoneally) induced rather strong hyperactivity in mice. With pretreatment of 1.5 mg of CocH3/kg of body weight (intraperitoneally), the mice had slight hyperactivity between 2 and 5 min and then returned back to the baseline level of activity. With pretreatment of 2 mg of CocH3/kg of body weight (intraperitoneally), 25 mg of cocaine/kg of body weight (intraperitoneally) did not induce any significant hyperactivity in mice, suggesting that the minimum dose of CocH3 required to completely block the hyperactivity induced by 25 mg of cocaine/kg of body weight (intraperitoneally) was 2 mg of cocaine/kg of body weight.

We further increased the dose of cocaine to 90 mg/kg of body weight (LD50 for intraperitoneally injected cocaine), and noted that 90 mg of cocaine/kg of body weight (intraperitoneally) did not induce any significant hyperactivity (or any sign of toxicity in our further observation after the hyperactivity tests) in the mice pretreated with 5 mg of CocH3/kg of body weight as seen in Figure 7. The observation suggests that the physiological effect of such a lethal dose of cocaine can be blocked completely by pretreatment of 5 mg of CocH3/kg of body weight.
In this way, a therapeutic enzyme could reduce cocaine’s entry into the brain to an amount (threshold) that is too low to produce detectable physiological effects. When the cocaine concentration in brain does not reach the ‘threshold’ value because of the presence of an efficient enzyme in plasma, one may consider that the enzyme has effectively blocked cocaine from reaching the brain. Furthermore, the threshold concentration of cocaine in brain is related to the degree of the dopamine transporter occupancy by cocaine. Volkow et al. [28] demonstrated that, for humans, “at least 47% of dopamine transporters have to be blocked for subjects to perceive cocaine’s effects”. The threshold concentration of cocaine in brain required to produce physiological effects was estimated to be $0.22 \pm 0.07 \mu M$ in the light of a recently reported cocaine pharmacokinetic modelling [29]. The data in Figure 7 indicate that, in the presence of the exogenous cocaine hydrolase (5 mg/kg of body weight), cocaine can be degraded so rapidly that even a lethal dose of cocaine (90 mg/kg of body weight, intraperitoneally) did not produce a brain cocaine concentration greater than the threshold (0.22 $\pm 0.07 \mu M$) required to induce detectable physiological effects; the animals did not show any detectable behavioural abnormality. The data indicate that it is possible to completely eliminate cocaine’s physiological effects by administration of a highly efficient cocaine hydrolase, providing a proof of the principle for the desirable enzyme therapy for cocaine abuse treatment.

According to previous studies [30], one-time use of cocaine could increase dopamine transporter (the primary target protein for cocaine) expression on the cell surface for months so that it takes a long time for the brain’s communication system to return to normal. Repeated administration of cocaine would reinforce the effects on the expression levels of transporters/receptors and continuously change the circuits in the brain. Hence, for effective treatment of cocaine addiction, it is essentially important to completely protect the brain from the cocaine effect so that the brain can gradually recover to function normally. It would be interesting to further test the cocaine hydrolase in a well-established animal addiction model including cocaine self-administration in the near future.

Conclusion

In the present study, we have successfully developed a stable cell line efficiently expressing a promising cocaine hydrolase, CocH3 (the A1995S/F227A/S287G/A328W/Y332G mutant of human BChE, with a yield of 1.4 pg/cell per day), by using a lentivirus-based repeated-transduction method. This is the first report to use a lentivirus vector to express human BChE or its mutant. The scaled-up protein production enabled us to characterize the cocaine hydrolase in rodents with regard to the biological half-life and potency in accelerating cocaine clearance. In particular, it has been demonstrated that the exogenous enzyme (cocaine hydrolase) can rapidly metabolize cocaine and completely eliminate cocaine-induced hyperactivity in rodents. The administration of CocH3 can completely eliminate the physiological effects of cocaine, providing a proof of the principle for a desirable enzyme therapy using CocH3 for cocaine abuse treatment.

AUTHOR CONTRIBUTION

Liu Xue prepared the lentivirus encoding the gene of the enzyme for development of stable cell lines, infected CHO-S cells with the lentivirus, performed HPLC analysis and the in vivo studies, and drafted the paper. Shuorong Hou performed the in vivo studies together with Liu Xue. Min Tong prepared the lentiviral vector and infected the cells with the lentivirus at an early stage of the work. Lei Fang ran the activity-stained non-denaturing polyacrylamide gel and participated in the locomotor activity experiments. Xiabin Chen participated in the HPLC analysis. Zhenyu Jin performed cell culture and protein purification. Hsin-Hsung Tai supervised Min Tong for the work related to lentivirus and stable cell lines. Fang Zheng and Chang-Guo Zhan designed the project, and Chang-Guo Zhan finalized the paper.

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