A Cocaine Hydrolase Engineered from Human Butyrylcholinesterase Selectively Blocks Cocaine Toxicity and Reinstatement of Drug Seeking in Rats

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Successive rational mutations of human butyrylcholinesterase (BChE) followed by fusion to human serum albumin have yielded an efficient hydrolase that offers realistic options for therapy of cocaine overdose and abuse. This albumin-BChE prevented seizures in rats given a normally lethal cocaine injection (100 mg/kg, i.p.), lowered brain cocaine levels even when administered after the drug, and provided rescue after convulsions commenced. Moreover, it selectively blocked cocaine-induced reinstatement of drug seeking in rats that had previously self-administered cocaine. The enzyme treatment was well tolerated and may be worth exploring for clinical application in humans.

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INTRODUCTION

Cocaine abuse and dependence in the US are problems with devastating medical and social consequences (Schrang, 1992; Marzuk *et al*, 1995), but there is no reliable means to treat cocaine overdose or reduce the likelihood of relapse in users who have achieved abstinence. Human plasma butyrylcholinesterase (BChE) contributes to normal cocaine metabolism (Inaba *et al*, 1978) and has been considered for use in treating cocaine toxicity (Gorelick, 1997).

Opposing or reversing the physiological and behavioral effects of cocaine with human BChE has become more feasible, as mutagenesis efforts have enhanced the cocaine hydrolase (CocH) activity of this enzyme (Figure 1). Our research began with molecular dynamics simulation and computer-based ligand docking to guide mutations (A328W/Y332A) that elevated the BChE rate constant for cocaine hydrolysis (k_{cat}) by a factor of 40 (Sun *et al*, 2001, 2002a). The double mutant blocked cocaine-induced locomotor activity in mice, blunted pressor responses in

rats, and reduced drug accumulation in brain tissue (Sun *et al*, 2002b; Gao and Brimijoin, 2004). Further mutations identified by Pancook *et al* (2003) using random mutagenesis raised k_{cat} by another factor of 5. Pan *et al* (2005) then simulated the BChE-cocaine transition state and devised a means to lower its free energy by combining the mutations, A199S/S287G/A328W/Y332G. These four substitutions can be said to yield a true CocH with a catalytic efficiency that is 1000-fold greater than wild-type BChE.

We wished to determine whether such a powerful catalytic tool might have substantial abilities to blunt cocaine toxicity and reduce or eliminate the central effects associated with cocaine-seeking behavior. To obtain a form of CocH that might be developed as a protein therapeutic, we fused this recombinant BChE at its C terminus with human serum albumin. Similar fusion proteins have been observed to possess favorable pharmacokinetic properties with high stability and extended plasma half lives (Duttaroy *et al*, 2005). In work that is being reported elsewhere (Gao and Brimijoin, unpublished results), we observed that the mutant BChE-albumin fusion, 'Albu-CocH' retains high catalytic efficiency with cocaine ($k_{\text{cat}} = 2700 \text{ min}^{-1}$, $K_m = 2 \mu\text{M}$) and exhibits a plasma half-life of 8 h after i.v. injection to rats. Here, we describe the effects of Albu-CocH on cocaine metabolism and toxicity as well as the cocaine-primed reinstatement of drug-seeking behavior in rats with a history of cocaine self-administration.

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Figure 1 Catalytic power of wild-type BChE (WT) and CocHs derived from this enzyme. Values from the published literature are expressed as k_{cat} (molecules of natural, (–)-cocaine hydrolyzed per min per molecule of enzyme). Amino-acid substitutions in the BChE mutants are A328W/Y332A (CocE; Sun *et al*, 2002a,b); F227A/S287G/A328W/Y332A (AME₃₅₉; Pancook *et al*, 2003); A199S/S287G/A328W/Y332G (CocH; Pan *et al*, 2005).

MATERIALS AND METHODS

Animals

Animals were handled according to the Principles of Laboratory Animal Care (National Research Council, 2003) in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care, under IACUC protocols A9306 (Mayo Clinic) and 0410A64760 (University of Minnesota). Wistar rats were obtained from Harlan Sprague–Dawley (Madison, WI). Female rats (20, weighing ~225 g at study onset) were used for tests on drug-primed reinstatement, as they are more sensitive to this behavioral effect than males and represent a greater challenge to potential therapy (Lynch and Carroll, 2000). Sex was not systematically investigated as a behavioral variable due to the extensive training required for the reinstatement experiments and the need to economize on enzyme. Toxicological and biochemical experiments largely used male rats (103, weighing 250–300 g), but 20 females (200–250 g) were included to confirm the generality of selected outcomes. Unless specifically indicated, the animals were not subjected to multiple procedures or used for more than one experiment.

On arrival at the behavior laboratory, rats were pair-housed in plastic cages and allowed to acclimate at least 3 days. Initially, all rats had free access to food (Purina Laboratory Chow, Purina Mills, Minneapolis, MN) and water. During the experiments, they received 16 g of food at 1500 hours daily, a procedure that was previously found to maintain animals at 85% of the free-feeding weight of age-matched controls. This modest food restriction reduces the variability in cocaine self-administration by equating food intake (a variable that is related to drug intake) across animals. It has been standard in our previous studies of cocaine-rewarded behavior (Carroll and Comer, 1996). Room lights were on from 0600 to 1800 hours. Temperature (24°C) and humidity (40–50%) were kept within a narrow range. The rats for locomotor study remained pair-housed,

except during the testing procedures (activity at 0900–0930 hours; food reinforcement at 1300–1500 hours). For the reinstatement task rats were permanently housed in operant testing chambers (see Behavioral apparatus).

Drug, Reagents, and Enzymes

Drugs were prepared in 0.9% NaCl (saline). Cocaine HCl was from Mallinckrodt (St Louis, MO). Other drugs including atropine sulfate, ketamine, amphetamine sulfate, di-isopropylfluorophosphate (DFP), and sodium pentobarbital were from Sigma Aldrich (St Louis). Albu-CocH is a fusion of truncated (E1–V529) mutant BChE (accession number gi:116353) to the N terminus of albumin (gi:28592). As described fully elsewhere, monomeric protein was expressed in Chinese hamster ovary cells and purified to near homogeneity (>95%) using blue affinity and ion exchange chromatography. Each batch of enzyme was titrated by incubation for 24 h with varying amounts of the irreversible inhibitor, DFP, followed by determination of residual activity (Sun *et al*, 2002a). For control experiments that required permanently inactivated enzyme, aliquots of Albu-CocH were incubated for 24 h at room temperature with DFP, 10^{-4} M, and then dialyzed overnight at 4°C against 50 mM sodium phosphate buffer, pH 7.4.

Radiometric Assay of Plasma and Tissue Samples

To assess drug metabolism, rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and given 3 H-cocaine (3.5 mg/kg, 30 μ Ci, i.v.). At 30 s to 2 h after rats been anesthetized, blood (~150 μ l) was collected from the tail or femoral veins into heparin-treated tubes containing DFP (10 μ l, 10^{-2} M), added to inhibit BChE and carboxylesterase. The samples were centrifuged (10 min at 8000 g) to obtain plasma, which was frozen on liquid nitrogen for assay later that day. Brains and hearts, obtained and frozen after aortic perfusion with 100 ml of isotonic NaCl plus DFP, 10^{-5} M, were later homogenized in phosphate-Tween buffer, also with DFP. In a solvent-partitioning procedure previously validated by mass-spectrometry (Brimijoin *et al*, 2002), aliquots for determination of cocaine (acidified with 1 ml of 0.02 N HCl) or benzoic acid (alkalinized with 300 μ l of 1 M Na₂CO₃) were extracted directly into toluene fluor for scintillation counting.

Blood Pressure Recording

For continuous monitoring of blood pressure, rats were anesthetized with urethane (1.45 g/kg, i.p.). A sterile PE-50 cannula was then placed in one femoral artery and connected to a calibrated pressure transducer (Gould TA240). Body temperature was maintained by a heat lamp. Animals were stabilized for 30 min before drug was given.

Toxicity of Cocaine Overdose

Rats were dosed with i.p. cocaine from 30 to 1000 mg/kg. A small cohort received amphetamine sulfate instead. Cocaine at 100 mg/kg had been found regularly lethal in unprotected animals. Death was not an endpoint for the present studies, which instead used seizures as the primary index of serious

toxicity. It was not possible to blind the observer as to treatment. Pupil dilation was measured semiquantitatively (≤ 1 mm, 0; ~ 1.5 mm, +; 1.5–2.5 mm, ++; > 2.5 mm, +++). In a battery of observations that continued at 5-min intervals for 60 min (with a 24 h check), the following toxic signs were scored as present or absent: piloerection, excessive locomotor activity (ie, running or ceaseless circling of the cage), labored breathing, persistent and marked head bobbing, ataxia (highly unstable gait), and prostration. Convulsions were noted and their onsets and durations were recorded. Rats were euthanized (sodium pentobarbital, 250 mg/kg i.p.) if they convulsed once for 60 s or twice within 2 min.

Behavioral Apparatus

The operant chambers were custom octagonal units with alternating panels of stainless steel or Plexiglas and a steel grid floor. They contained two response levers with stimulus of lights above them (a ceiling light), a food pellet dispenser, and a water bottle mounted outside. Tygon tubing connected a tether and swivel at the top of the cage to a syringe pump mounted outside a wooden enclosure that surrounded the test chamber. Data collection and experimental programming were controlled by MED-PC software (Med Associates, St Albans, VT).

As previously described (Perry *et al*, 2005), the locomotor track was a circular stainless steel of 71 cm diameter. It was equipped with four infrared sensors around the inner perimeter, at 0°, 90°, 180° and 270°, and connected to a VersaMax programmable logic controller (IC200UDR001, GE Fanuc Automation, Charlottesville, VA).

Surgical Preparation for Cocaine Self-administration

Under anesthesia with ketamine (60 mg/kg) and xylazine (10 mg/kg), with atropine (0.15 ml) and doxapram (5 mg/kg) to facilitate respiration, 15 rats were implanted with a 15 cm silastic catheter (0.51 mm i.d., 0.94 mm o.d.; Helix Medical Inc., Carpinteria, CA). The catheter, with two beads of prosthetic silicone elastomer, 3 and 3.5 cm from one end (MDX4-4210; Factor II Inc. Lakeside, AZ), was introduced into the right jugular vein and anchored with sterile silk sutures. The free end was led to an exit incision medial and 1 cm caudal to the scapulae. Heparin (10 IU/kg, i.v.) and gentomycin (12.0 mg/kg, i.v.) were administered for 3 days to prevent clotting.

Behavioral Training and Reinstatement

The catheterized rats were trained to lever press under a fixed ratio 1 (FR 1) schedule, which delivered one infusion of cocaine contingent upon one lever-press. Behavioral sessions were conducted from 1300–1500 hours daily. When responding had stabilized for 14 days, cocaine was replaced with physiologic saline. Self-administration behavior was then allowed to extinguish for 21 days, during which responses continued to produce infusion-related stimuli and saline infusions. Next followed a 3-day cue-extinction period in which the infusion pump and house light were disconnected to completely extinguish responding and ensure that reinstatement specifically reflected drug-priming cues.

Subsequent reinstatement sessions involved no self-administration of cocaine or saline, or infusion-related stimuli. To begin each session, the experimenter administered an i.p. priming injection of saline (S), cocaine (C), or amphetamine (A) daily at 1300 hours for 12 days according to the following sequence: SCSCSASASCSA. On the 4th (C) and 6th (A) day, the rats were pretreated at 0900 hours with 2 mg/kg (i.v.) Albu-CocH, administered through the infusion apparatus and flushed with 0.3 ml sterile saline.

Locomotor Activity and Food Reinforced Behavior

Locomotor activity was assessed in a different group of five rats daily for 30 min beginning at 1100 hours by detecting infrared beam breaks, as described by Perry *et al*. (2005). Two or more breaks of one beam occurring before another beam was broken were counted as a single response. After behavior was stabilized (no steadily increasing or decreasing trends for 3 days), rats were injected at 0900 hours with saline (tail vein) for 4 days, then Albu-CocH for 1 day and saline again on the final day.

These rats were then placed in the operant conditioning chambers for a 3-h food session at 1300 hours daily. Food pellets (45 mg) were contingent upon responses on either lever under a FR 1 adjusting delay discounting schedule (Perry *et al*, 2005). A response on the 'immediate lever' produced one pellet immediately, whereas a 'delay lever' response resulted in three pellets after a delay that altered during the session based on the animal's behavior. The delay started at 6 s, and it increased by 1 s after a 'delay lever' response and decreased by 1 s after an 'immediate lever' response. The session ended on completion of 60 trials or after 3 h, whichever came first. Following the session, rats were given additional food to reach a total of 16 g per day. Each day the delay started at the value it ended with the day before.

Each session was divided into 15 four-trial blocks. The first and second trials of each block were forced exposure to each lever (immediate and delayed condition in counter-balanced order), whereas the third and fourth trials were free choice, immediate or delayed, and a response on either lever yielded one or three pellets, respectively. A mean adjusted delay (MAD) was calculated by averaging the delays that were in effect on all of the free choice trials (maximum = 30) completed on the 'delay' lever. MAD values served as a quantitative measure of impulsivity for food and provided another dimension of food-rewarded behavior in addition to amount of food earned.

Statistical Analysis and Pharmacokinetic Calculations

Data were analyzed statistically with StatView 4.5 (Abacus Concepts, Berkeley, CA). Toxicity scores were evaluated with Chi-square tests. Treatment effects on blood pressure were subjected to repeated-measures analysis of variance (ANOVA) with time and treatment as factors. Factorial ANOVA was used in endpoint studies on brain cocaine levels to evaluate effects of treatment, sex, and interactions. Self-administration and other behavioral response data were analyzed by one- and two-way ANOVA, followed by *post hoc* testing. Data were reported as means $\pm$ SE; $p < 0.05$ was considered statistically significant.

RESULTS

When purified Albu-CocH was injected through the tail vein into otherwise untreated male Wistar rats (250–300 g, 4 rats per dose), it had no outwardly discernable effects at 1 or 3 mg/kg, whereas 10 mg/kg caused mild lethargy for about 1 h. In anesthetized animals, Albu-CocH at 3 mg/kg unexpectedly caused a modest rise in blood pressure, on the order of 10 mm Hg, which lasted approximately 5 min (Figure 2). In contrast, the same dose of enzyme greatly reduced pressor responses to a moderate dose of cocaine administered 10 min later. This antipressor effect was specific to cocaine, because Albu-CocH did not reduce the hypertensive response to norepinephrine (Figure 2).

The blood pressure findings led us to hypothesize that Albu-CocH would be able to alleviate the toxicity of a major cocaine overdose. Testing that possibility in rats required the exposure of awake, unrestrained animals to doses of cocaine with a potential to evoke serious adverse effects (defined as seizures). Our experience had shown that an i.p. challenge with cocaine (100 mg/kg) regularly induced convulsions that ended in death within 2 min unless euthanasia was administered.

Table 1 summarizes observations on a total of 44 male and female rats entered on this protocol. After receiving the cocaine challenge, each of the 15 unprotected rats developed hyperlocomotion, then ataxia, and then convulsions (onset time, 170 ± 30 s), which met the euthanasia criterion. Female rats were approximately as sensitive as the male subjects, particularly with regard to seizures and other major toxicity. For economy with both animals and enzyme, the initial experiments with enzyme treatment were conducted in a single sex (males). Pretreatment with i.v. Albu-CocH provided dramatic, dose-dependent protection against cocaine. A small dose (1 mg/kg) delayed but did not prevent arousal or seizures in four of the four rats (onset, 380 ± 70 s); a mid-dose (3 mg/kg) prevented seizures but not signs of arousal (six out of six); a large dose (10 mg/kg) eliminated arousal and seizures (six out of six), and it raised cocaine's ED_{50} for this toxicity nearly 10-fold (Figure 3).

Accelerated cocaine hydrolysis is the simplest explanation for this protection, because the same cocaine challenge caused convulsions that required euthanasia of five sham-treated animals (three males given 10 mg/kg human serum albumin and two given Albu-CocH inactivated by DFP). The protection was specific to cocaine, as active enzyme at 10 mg/kg failed to delay or prevent convulsions in three male rats challenged with amphetamine at the threshold dose (150 mg/kg) that produced uniform seizures in our unprotected rats. Protection against cocaine was lasting. As Table 1 shows, no seizures occurred in any of eight rats challenged up to 12 h after receiving 10 mg/kg Albu-CocH, and partial protection remained at 24 h.

Human overdose requires rescue. To evaluate rescue potential, we injected 100 mg/kg cocaine i.p. into 17 rats (8 females and 9 males), waited for onset of convulsions, then rapidly administered Albu-CocH (Table 2). Each of the six rats given 10 mg/kg Albu-CocH ceased convulsing within 1 min, resumed an upright posture within 2 min, and showed limited further signs of cocaine-induced arousal. After 20 min, apart from lethargy and mild paw swelling for 1–2 h, treated rats resembled untreated

Figure 2 Selective blunting of cocaine-induced hypertension. Blood pressure was recorded from the femoral artery in 22 male rats under urethane anesthesia (1.45 g/kg). After 30 min, Albu-CocH (filled circles, 3 mg/kg, i.v., $n=5$) or saline (open circles, $n=13$) was administered, followed by atropine (1 mg/kg) to reduce vagal reflexes, and baseline pressure was recorded for 10 min. Enzyme alone caused a small rise in blood pressure (top panel). At 10 min, the rats were challenged with 3.5 mg/kg cocaine (middle panel). Ten minutes later, the five enzyme-treated rats were re-challenged with 6 µg/kg norepinephrine (NE, bottom panel), which was also administered to a control group of four atropine-treated animals with no prior cocaine or enzyme exposure (bottom panel). Changes in mean blood pressure are shown. Repeated measures ANOVA showed that the pressor effects evoked by cocaine and NE were both significant ($p < 0.001$), as was the Albu-CocH-induced reduction of the cocaine effect ($p < 0.01$).

controls. All eight rats given 3 mg/kg Albu-CocH also ceased convulsing within 1 min and quickly regained upright posture. One of the five treated females experienced two additional short seizures and died after 6 min. The other animals had no further seizures, but for approximately 20 min, most of them exhibited signs of arousal, including maximally dilated pupils, head bobbing (in males), and hyperlocomotion. On the next day, both treated groups were indistinguishable from rats that never received cocaine. At 10 mg/kg, Albu-CocH was also partially effective against a larger cocaine overdose, saving four of six male rats challenged with 300 mg/kg of i.p. cocaine (data not shown). In contrast, wild-type BChE (3 mg/kg) was

Table 1 Albu-CocH Protects Against Cocaine Toxicity

Agent	None		Albu-CocH					
Sex	Female Male		Male only					
Enzyme dose	0	0	1 mg/kg	3 mg/kg	10 mg/kg			
Challenge delay	None	None	10 min	10 min	10 min	1–6 h	12 h	24 h
Number of rats	4	11	4	5	6	4	4	6
Percent of subjects displaying specific toxic signs								
Pupils +	100	100	100	100	100	100	100	100
Pupils ++	100	100	100	100	17	0	0	100
Pupils +++	100	100	100	60	0	0	0	50
Piloerection	100	100	100	0	0	0	50	100
Hyperlocomotion	60	91	100	100	17	100	100	82
Hard breathing	100	100	100	100	100	0	50	100
Head bobbing	0	55	100	100	0	100	100	82
Ataxia	100	100	100	0	0	0	25	67
Prostration	100	100	100	0	0	0	0	50
Seizure	100	100	100	0	0	0	0	67
Recovery	0	0	25	100	100	100	100	67*

A total of 44 rats were challenged with cocaine, 100 mg/kg, i.p., at the indicated times after pretreatment with Albu-CocH in different doses. Toxic signs were defined, noted, and scored as described under Materials and Methods. Most animals experiencing seizures died rapidly. Prolonged or recurrent seizures were grounds for euthanasia. Recovery involved complete reversal of all observed adverse effects, usually within 1 h and always within 24 h. Statistical significance of therapeutic effects was tested by χ^2 analysis. Data from the 11 unprotected males and 4 unprotected females (which did not differ significantly from each other) were pooled. Figures in bold represent statistically significant differences from the pooled controls ($p < 0.01$, except where indicated by * $p < 0.05$).

ineffective in rescuing three of three male rats even after standard cocaine challenge (100 mg/kg), all of which met our criterion for early euthanasia (Table 2).

These results established dose-dependent and hydrolase-specific rescue from cocaine intoxication. To test the hypothesis that the rescue involved accelerated cocaine metabolism, we carried out two different experiments using radiometric assays to follow tissue levels of ^3H -cocaine and its metabolite ^3H -benzoic acid. The first experiment, with eight male rats, examined Albu-CocH pretreatment. In four controls treated with ^3H -cocaine (3.5 mg/kg, i.v.), plasma drug half-life was 50 ± 5 min, while in four other rats given 3 mg/kg Albu-CocH 10 min beforehand, 98% of the free drug was converted into benzoic acid within 30 s (Figure 4), and the drug burden in heart and brain was greatly reduced (Figure 5). Thus, cocaine was rapidly destroyed if injected when the enzyme was present.

To further elucidate the rescue phenomenon, a second experiment was conducted to determine whether Albu-CocH could remove cocaine previously accumulated in the central nervous system. For that purpose, in a balanced design with eight female and eight male rats, we reversed the order of events and administered ^3H -cocaine 10 min before enzyme. Brains were then harvested after a further 10 min. These times were chosen in light of data from naïve rats indicating that brain cocaine levels declined exponentially with an estimated half life of 9 ± 1.4 min during the

Figure 3 Effect of Albu-CocH (10 mg/kg) on the dose–response curve for seizures from different amounts of cocaine administered 10 min later. A total of 53 male rats were studied (data for those that received cocaine 100 mg/kg are also reported in Table 1). Values beside data symbols represent number of animals per group.

first 20 min after i.v. drug administration (Figure 6a). The effect of Albu-CocH on brain cocaine in the delayed-treatment paradigm was unequivocal (Figure 6b and c). In rats of both sexes, this treatment reduced the final levels of ^3H -cocaine approximately fourfold and caused substantial increases in ^3H -benzoic acid. These data directly demonstrate enzyme-driven drug elimination from brain.

An enzyme powerful enough to rescue rats from cocaine toxicity might also be useful in reducing drug-reward and managing cocaine addiction. To evaluate that possibility, we tested Albu-CocH in rats trained to self-administer cocaine. One of the most refractory aspects of cocaine addiction is relapse after abstinence. Our goal was to determine whether fast metabolism of cocaine en route to brain reward centers could prevent relapse triggered by an i.v. priming injection of cocaine. More specifically, we examined the effect of Albu-CocH pretreatment on the cocaine-primed reinstatement of drug-seeking behavior in 15 female rats that had previously self-administered cocaine and subsequently extinguished their responding when saline replaced cocaine.

The rats were trained to emit one lever press for each cocaine infusion (0.4 mg/kg, i.v.) during daily 2-h sessions under a fixed-ratio 1 (FR 1) schedule. After behavior stabilized for 14 days, saline was substituted for cocaine for 21 days, and behavior was allowed to extinguish. In a subsequent reinstatement phase, priming injections of cocaine were given (alternating daily with saline-priming injections). On selected days, rats received Albu-CocH (2 mg/kg) 2 h before the reinstatement session. Cocaine-priming injections (10 mg/kg, i.p.) generated 30–40 responses on the 2 days with no pretreatment (Figure 7). After Albu-CocH, however, cocaine priming resulted in negligible responding. Saline priming on intervening days also resulted in minimal responses (2–5) on the lever previously associated with cocaine. To control for possible nonspecific behavioral suppression, we also tested priming injections of d-amphetamine, which is not a hydrolase substrate. Amphetamine-priming injections (2 mg/kg, i.p.) elicited approximately 60 reinstatement responses, which, consistent with an effect that depended on selective metabolism, were not significantly reduced by Albu-CocH.

Table 2 Albu-CocH Rescues from Cocaine Overdose

Sex	Females				Males				
Enzyme dose	Albu-CocH 3 mg/kg		Albu-CocH 10 mg/kg		Albu-CocH 3 mg/kg		Albu-CocH 10 mg/kg		WT 3 mg/kg
Number of rats	5		3		3		3		3
Time window (min)	1–20	20–60	1–20	20–60	1–20	20–60	1–20	20–60	1–20
Percent of subjects displaying specific toxic signs									
Pupils +	100	100	100	100	100	100	100	100	100
Pupils ++	100	100	100	33	100	67	33	0	100
Pupils +++	100	100	100	0	100	0	0	0	100
Piloerection	100	20	0	0	33	0	0	0	100
Hyperlocomotion	60	0	67	0	67	0	0	0	a
Hard breathing	100	0	100	0	100	0	67	0	100
Head bobbing	0	0	0	0	100	0	0	0	a
Ataxia	40	0	0	0	33	0	0	0	100
Prostration	20	0	0	0	33	0	0	0	100
Seizure	20	0	0	0	0	0	0	0	100
Recovery		80		100		100		100	0

A total of 17 rats received cocaine, 100 mg/kg, i.p. Enzyme treatments in the indicated doses were administered i.v. immediately after full-blown clonic-tonic seizures had developed. Signs of toxicity were then observed at frequent intervals until the animals made a full recovery, died, or required euthanasia. Observations are summarized separately for the time windows from 1–20 min and 20–60 min after rescue treatment. Statistical significance of rescue effects was tested by χ^2 . Because no sex differences were apparent, data from enzyme-rescued rats were compared with pooled data from the 15 males and females that received no Albu-CocH either before or after the cocaine challenge. Bold figures represent significance, with $p < 0.01$ when treated males and females were pooled, and $p < 0.05$ when they were separately compared with the pooled controls. ^aRats given wild-type BChE remained moribund and did not show hyperlocomotion or head bobbing.

Figure 4 Accelerated cocaine clearance. Plasma cocaine levels are shown as a function of time after injection of cocaine ($30\mu\text{Ci}$, 3.5 mg/kg , i.v.) into male rats that 10 min earlier had received Albu-CocH (3 mg/kg , i.v. (black symbols), $n=4$) or saline (empty symbols, $n=4$). Blood samples were drawn from the femoral vein beginning 30 s after cocaine and were assayed radiometrically. As shown here, plasma cocaine levels in control rats declined slowly but in Albu-CocH-treated rats they dropped nearly to the detection limit by the earliest sampling point (30 s after drug injection, $p<0.001$).

To further confirm that Albu-CocH did not cause generalized behavioral suppression that might impair reinstatement after cocaine priming, we also investigated the effect of Albu-CocH on locomotor activity and responding for food. For these studies, five female rats were treated alternately with i.v. saline, Albu-CocH, and saline. Two hours after each day's injection, locomotion was monitored in a circular open field with four infrared beams equally spaced around the perimeter (Piazza *et al*, 1989). The beam break data showed no treatment effect (Table 3).

Four hours after the saline or enzyme injections, the same rats were studied in an operant conditioning experiment with food delivery contingent upon FR 1 lever-press responding in a paradigm designed to assess impulsivity for reward (Perry *et al*, 2005). Results after Albu-CocH showed no significant differences (*vs* saline) in trials completed, number of pellets earned, food intake, or mean-adjusted delay for the 60 choice trials (Table 3).

DISCUSSION

Previous Development of Cocaine Hydrolases and Binding Proteins

The present study follows a decade of research into the therapeutic possibilities of agents that accelerate cocaine hydrolysis. This process began with the recognition that human BChE not only hydrolyzes cocaine but also accounts for much of its metabolism (Stewart *et al*, 1977; Inaba *et al*, 1978). BChE-catalyzed cocaine hydrolysis generates two breakdown products, benzoic acid and ecgonine methyl ester. Compared with cocaine and other metabolites, like norcocaine and benzylecgonine, these detoxified products have little biologic activity (Misra *et al*, 1975; Madden and Powers, 1990). A report that deficient plasma BChE activity increased the risk of toxicity after cocaine exposure (Hoffman *et al*, 1992) led to tests of BChE for prophylaxis.

Figure 5 Reduced tissue accumulation of cocaine. Male rats ($n=4$ per group) received ^3H -cocaine ($30\mu\text{Ci}$, 3.5 mg/kg , i.v.) 10 min after treatment with Albu-CocH (3 mg/kg , i.v.) or saline. Ten minutes after the cocaine injections, brains, hearts, and plasma were collected for analysis of cocaine and its metabolite, benzoic acid. Treatment with Albu-CocH greatly lowered tissue burden. Intact cocaine was nearly undetectable in hearts and plasma from the enzyme-treated rats, where it was quantitatively replaced by the metabolite, benzoic acid. The treatment effect was substantial in brain as well, but smaller, consistent with the fact that nervous tissue is a preferred site for cocaine uptake.

In one such test (Hoffman *et al*, 1996), large quantities of the enzyme (30 mg/kg) partially protected mice against a normally lethal dose of cocaine (150 mg/kg). Such findings lent credence to a general therapeutic strategy based on the binding and enhanced metabolic conversion of cocaine (Gorelick, 1997). As native human BChE has low catalytic efficiency with cocaine ($k_{\text{cat}} \approx 4\text{ min}^{-1}$) and only moderate affinity ($K_{\text{m}} \approx 3\mu\text{M}$), a more powerful agent was needed. Subsequent investigation has focused on (1) modified versions of BChE, (2) natural cocaine esterases, and (3) antibodies, especially those exhibiting catalytic activity.

Seeking a catalytic immunoglobulin, Landry *et al* (1993) raised an antibody against a transition-state analog that weakly hydrolyzed cocaine. An improved antibody, mAB 15A10, reduced the toxicity and rewarding effect of cocaine in rats (Mets *et al*, 1998; Baird *et al*, 2000; Briscoe *et al*, 2001). That outcome was surprising, because the antibody bound and hydrolyzed cocaine more weakly than BChE itself, and cocaine was given in large molar excess. The most active cocaine antibody reported to date (Cashman *et al*, 2000) in fact compares favorably with native BChE (10-fold greater k_{cat} , 5-fold higher affinity). Although the therapeutic

Figure 6 Albu-CocH treatment accelerates removal of cocaine depots in brain. **(a)** Time course of radiolabeled cocaine in brains of 13 untreated or saline-treated male rats given 3.5 mg/kg ^3H cocaine, i.v., at time zero. Cocaine cpm/g wet weight of cerebral cortex is shown. **(b)** and **(c)**. Enzyme-driven removal of brain cocaine. Eight male and eight female rats received radiolabeled cocaine, as above, and 10 min were allowed for redistribution and brain uptake. Saline (control) or 10 mg/kg Albu-CocH (enzyme) were then given and, after another 10 min, samples of cerebral cortex were obtained and analyzed for radiolabeled cocaine and benzoic acid. Shown are counts per min (cpm) per 20 mg of brain or 50 μl of plasma. In rats of both sexes the large enzyme-induced reductions of the parent drug and increases of the metabolite were highly significant ($p < 0.001$) by factorial ANOVA. The analysis indicated that actual levels of brain cocaine were slightly higher in females than in males ($p < 0.02$), but there was no significant sex difference in benzoic acid, nor was there a sex-by-treatment interaction.

potential of that antibody has not been evaluated, non-catalytic cocaine antibodies of similar affinity have been found to moderately reduce cocaine toxicity (Carrera *et al*, 2005). It is therefore conceivable that anti-cocaine

Figure 7 Selective blockade of cocaine-primed reinstatement of drug-seeking behavior. Fifteen rats that had previously self-administered cocaine and extinguished when cocaine was replaced with saline were primed with an i.v. injection of saline (S), cocaine (C, 10 mg/kg), or amphetamine (A, 2 mg/kg) just before each of 12 daily, 2-h sessions. On days 4 and 6, they received Albu-CocH enzyme (E, 2 mg/kg i.v., 2 h before their 2-h behavioral session. Data shown are mean $\pm$ SEM of total responses on the previously active lever (which had no consequences). Horizontal brackets indicate statistical comparisons (* $p < 0.05$; ** $p < 0.01$).

antibodies, administered directly or elicited by vaccination, will play some role in future therapy of cocaine abuse. Nonetheless, although efforts to produce more catalytically efficient antibodies continue (McKenzie *et al*, 2007), true enzymatic CoCHs represent a much more promising option.

A search for natural enzymes led to an efficient CoCH in *Rhodococcus sp.* MB1, a bacterium linked with coca plants (Bresler *et al*, 2000; Larsen *et al*, 2002; Turner *et al*, 2002). This enzyme can prevent lethal cocaine-induced seizures in rats and increase the dose for cocaine toxicity 10-fold (Cooper *et al*, 2006). Bacterial cocaine esterase deserves more study as a rescue agent. Issues of stability, time course, and antigenicity, however, present challenges to clinical application. Cocaine esterase is sufficiently stable for acute use, but its *in vivo* half-life in rats is less than 15 min. Furthermore, as a foreign protein, cocaine esterase can be expected to evoke immune responses in humans. In mice, anti-esterase antibodies appeared after three injections (Ko *et al*, 2007) and reduced the protective activity of further administrations. Chronic interventions with bacterial enzymes, therefore, are unrealistic.

Use of Human BChE

Human BChE offers advantages of high stability and lack of direct toxicity. Antibodies arise after heterologous administration of BChE, but not after homologous administration, within a given species (Maxwell *et al*, 1992). Large quantities of native BChE are well tolerated by all tested species (Lynch *et al*, 1997; Carmona *et al*, 2000; Doctor and Saxena, 2005). Mice with high circulating levels of human BChE showed no clinical signs, postmortem examinations revealed no abnormalities in serum chemistry or hematology (Saxena *et al*, 2006), and the acoustic startle reflex remained normal (Clark *et al*, 2005). Finally, daily administration of the partially purified wild-type protein for several weeks to human subjects has evoked no adverse

Table 3 Albu-CocH does not Alter Locomotor Activity or Food-Reinforced Responding

Injections	Locomotor	Food-reinforced behavior			
	Beam breaks	Trials completed	No. of pellets	Food intake (g)	mean adjusted delay ^a (sec)
Saline	23.8 ± 2.4	57.2 ± 2.8	110 ± 4.9	4.93 ± 2.2	14.0 ± 5.0
Albu-CocH	21.2 ± 4.2	57.8 ± 1.4	115 ± 6.2	5.15 ± 2.7	7.44 ± 0.45
Saline	21.0 ± 2.6	54.2 ± 5.8	108 ± 10.3	4.88 ± 4.6	6.84 ± 1.5

Five rats received saline or Albu-CocH at 0900 hours on 3 consecutive days. Mean values ($\pm$ SEM) are shown for locomotor activity (1100 hours) and food-rewarded behavior (1300 hours). The enzyme treatment had no statistically significant effect on any of the tested measures when examined by ANOVA.

^aSelf-determined measure of impulsivity for food.

effects (Cascio *et al*, 1988). The stability, antigenicity, and safety of Albu-CocH, on the other hand, still require extensive further investigation, particularly in humans. Meanwhile, it should be borne in mind that the key modifications in the catalytic unit involve amino-acid residues that have little surface exposure to immune surveillance. It is also worth noting that the great increase in catalytic activity against cocaine is not accompanied by a comparable effect on acetylcholine hydrolysis. In fact, in our own comparisons with wild-type BChE (Gao and Brimijoin, unpublished results), Albu-CocH exhibited a nearly 50% reduction in catalytic efficiency with acetylcholine. Thus, we would not expect systemic administration of this enzyme to disrupt cholinergic transmission in the periphery, and still less in the brain, where it is unlikely to penetrate.

Albu-CocH and Cocaine Toxicity

The protein engineering that led to CocH has fully overcome the initial limitation of poor catalytic efficiency in BChE (Pan *et al*, 2005) and placed it on a par with bacterial esterase in hydrolyzing cocaine. The BChE advantages of stability, low toxicity, and selective catalytic efficiency should be preserved in Albu-CocH, making it attractive as a protein-based therapeutic. The present results show that these properties combine with an ability to prevent cocaine access to critical biological targets, including heart and brain, and to block or reverse cardiovascular and neurological toxicity.

The approximate 10-fold rightward shift in the dose-response curve for seizure induction by cocaine after Albu-CocH is equivalent to the recently reported effect of bacterial cocaine esterase (Cooper *et al*, 2006). The bacterial enzyme lost its ability to protect in 30 min or less, but Albu-CocH treatment remained fully protective for 12 h. In both cases, protection required catalytic activity, and treatments were ineffective if the enzymes were inactivated in a manner expected to preserve cocaine binding. Our biochemical data support this concept by demonstrating powerful reductions in plasma and tissue cocaine levels when Albu-CocH was given before the drug.

We consider it particularly important that Albu-CocH was able to abort full-blown convulsive seizures in rats given large i.p. doses of cocaine. The rescue was rapid for an agent that should be largely excluded from the brain because of its high molecular weight (our unpublished data indicate that CocH activity in brain remains below 1% of

that in plasma). We infer that rescue injections create steep diffusion gradients favoring the loss of cocaine from sites throughout the body and especially from brain, with its high regional blood flow. This inference is consistent with our finding that enzyme treatment greatly reduced brain cocaine levels even when given minutes after drug administration, when redistribution from the plasma was approaching completion. The brain is a complex compartment, in which cocaine can be taken up by myelin lipids, captured by cells, and specifically bound to the sodium channels and amine transporters that comprise its primary targets. Further studies are required to determine the effects of Albu-CocH on cocaine adsorbed to such targets. Nonetheless, the present results indicate that an enzyme-driven elimination of cocaine in accessible compartments can shift the equilibrium in favor of dissociation, markedly and rapidly. Hence, efficient enzymes such as Albu-CocH may be well suited for the emergency treatment of cocaine overdose.

Intervention in Relapse or Reinstatement of Drug-seeking Behavior

Beyond emergency uses, the delivery of Albu-CocH might help addicts avoid the full relapse that commonly follows a brief lapse. Long plasma half-life is desirable for such a purpose. Plasma half-lives for therapeutic proteins invariably increase with the size of the animal (Mordenti *et al*, 1991). Allometric scaling from our rat data, in light of experience with other albumin fusion proteins (Osborn *et al*, 2002), is compatible with a several-day half-life of Albu-CocH in humans, allowing infrequent dosing. Gene transfer represents an alternative mode of delivery that, if perfected, could provide continuous supply. We have found that standard E1-deleted adenoviral vectors sustain effective levels of a CocH for days in the rat bloodstream (Gao and Brimijoin, 2005) and brain (Gao and Brimijoin, 2006). Ongoing work with a helper-dependent adenoviral vector is showing expression windows of several months (Gao and Brimijoin, unpublished data). Altogether, therefore, there is a fair prospect of being able to sustain conditions that may impede cocaine's ability to provoke relapse.

Relapse or 'reinstatement' is perhaps the greatest challenge in treating drug abuse (Kalivas and Volkow, 2005). Drug-seeking behavior in established animal models of reinstatement is regularly triggered by exposure to drug-associated environmental cues. Factors that predict or enhance reinstatement include female sex (Lynch and

Carroll, 2000), estrogen status (Anker *et al*, 2007; Larson *et al*, 2005), higher drug dose (Carroll and Campbell, 2000), addiction-prone phenotypes such as impulsivity (Perry *et al*, 2005) or sweet-preference (Perry *et al*, 2006), and food restriction (Comer *et al*, 1995). Especially powerful are 'priming exposures' of drugs with related pharmacological mechanisms (Carroll and Comer, 1996; Carroll and Campbell, 2000; Shalev *et al*, 2002; Shaham *et al*, 2003). Our experiments showed that Albu-CocH was fully effective in blocking reinstatement provoked by cocaine-priming injections. In contrast, under closely similar conditions, Albu-CocH did not affect amphetamine-primed reinstatement, locomotor behavior, food-rewarded behavior, or impulsivity for food.

In conclusion, the present results from an animal model of relapse support the hypothesis that, by preventing cocaine access to the brain, and/or reducing cocaine brain levels, accelerated metabolism can blunt not only toxicity but also the reward-seeking effects of this drug. The virtual elimination of cocaine-primed reinstatement suggests that sustained delivery of an efficient hydrolase would reduce the probability of relapse in recovering cocaine addicts even if it did not suppress craving. As BChE lacks obvious physiological effects and has been demonstrated safe in humans, Albu-CocH is a prime candidate for such a treatment.

DISCLOSURE/ CONFLICTS OF INTEREST

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