Rapid and Robust Protection against Cocaine-Induced Lethality in Rats by the Bacterial Cocaine Esterase

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ABSTRACT

There is no approved means to prevent the toxic actions of cocaine. Cocaine esterase (CocE) is found in a rhodococcal strain of bacteria that grows in the rhizosphere soil around the coca plant and has been found to hydrolyze cocaine in vitro. The esteratic activity of CocE (0.1–1.0 mg, i.v.) was characterized and confirmed in vivo by assessing its ability to prevent cocaine-induced convulsions and lethality in the rat. The therapeutic efficiency of the enzyme was demonstrated by the increasing dose of cocaine (100–1000 mg/kg, i.p.) required to produce toxic effects after a single intravenous injection of CocE. The enzyme demonstrated rapid kinetics for cocaine degradation in rat and human serum. Two catalytically inactive mutants of CocE (S117A or Y44F) failed to protect rats from the toxic effects of cocaine, confirming the protective effects are due to hydrolytic activity. However, butyrylcholinesterase, an endogenous cocaine-hydrolyzing enzyme, was inactive (1.3–13 mg, i.v.) in this rat toxicity procedure. Furthermore, CocE did not block the lethality of WIN-35065-2 (560 mg/kg, i.p.), a cocaine analog that lacks the benzoyl ester moiety targeted by CocE. This characterization of CocE provides preliminary evidence that the enzyme could serve as a suitable antidote to cocaine toxicity in humans.

The addictive and lethal properties of cocaine, derived from the South American shrub *Erythroxylum coca*, are well established (e.g., Carroll et al., 1999). Cocaine acts to block the reuptake of the monoamines dopamine, norepinephrine, and serotonin, thus prolonging and magnifying the effects of these neurotransmitters in the nervous system (e.g., Benowitz, 1993). The local anesthetic effect of cocaine is attributed to sodium channel blockade (Bauman and DiDomenico, 2002). Cocaine toxicity is marked by both convulsions and cardiac dysfunction due to effects on neurotransmitter systems and myocardial sodium channel blockade (Bauman and DiDomenico, 2002; Wilson and Shelat, 2003). Because of cocaine's ability to readily cross the blood-brain barrier and its widespread effects on the central and peripheral nervous systems, death by cocaine toxicity is rapid, and it often results in "sudden death" (for review, see Bauman and DiDomenico, 2002).

The rapid and pleiotropic effects of cocaine present a complex problem for the treatment of acute toxicity (Carroll et al., 1999). One approach to reduce cocaine's effects would be to increase its rate of degradation by administering either an anticocaine catalytic antibody (Landry et al., 1993; Carroll et al., 1999; Larsen et al., 2002) or an endogenous esterase such as butyrylcholinesterase (BChe).

The anti-cocaine catalytic antibody, monoclonal antibody 15A10, elicited by means of a phosphonate monoester transition-state analog for benzoyl esterolysis ($k_{cat} = 0.038$ s^{-1} and $K_M = 220$ μM), cleaves cocaine to yield ecgonine methyl ester and benzoic acid. These metabolites are nontoxic in mammals. mAb 15A10 is reported to decrease intravenous cocaine self-administration but to not suppress food-maintained response in rats (Baird et al., 2000). In addition, the catalytic antibody offered some protective effect against cocaine-induced lethality and hypertension for low doses of cocaine in catecholamine-sensitized rats (Mets et al., 1998; Deng et al., 2002). However, these effects were observed only

ABBREVIATIONS: CocE, cocaine esterase; PBS, phosphate-buffered saline.

at extremely high doses of the antibody (15–50 mg/kg) because of its low catalytic efficiency.

BChe is an endogenous protein synthesized in the liver of vertebrates that also hydrolyzes cocaine to yield benzoic acid and ecgonine methyl ester (e.g., Benowitz, 1993; Knuepfer, 2003). Treatment with exogenous BChe has been shown to decrease the cardiovascular and psychomotor stimulating effect of cocaine in rats and mice (Lynch et al., 1997; Mattes et al., 1997; Carmona et al., 1998; Koetzner and Woods, 2002). Furthermore, pretreatment with BChe offers some protection against the lethal effects of cocaine in rats and mice (Hoffman et al., 1996; Lynch et al., 1997). Coadministration of cocaine with BChe decreases the half-life of cocaine and increases plasma levels of the cocaine metabolites ecgonine methyl ester and benzoic acid, in both rodents and monkeys, demonstrating that its inhibition of cocaine's physiologic and behavioral effects is due to the cocaine-hydrolyzing capabilities of the enzyme (Carmona et al., 2000; Koetzner and Woods, 2002). The enzyme has also been shown to have a long half-life in rodents and monkeys (24–620 h), probably because it is produced endogenously and is, therefore, stable under physiologic conditions (for review, see Gorelick, 1997). Despite the catalytic properties and long half-life of BChe, the enzyme is not necessarily a viable therapeutic candidate for cocaine toxicity, primarily because of inadequate catalytic efficiency (k_{cat} = 3.9 min^{-1} and K_M = 14 μM) (Xie et al., 1999). Pretreatment with BChe 10 min before an i.v. or i.p. injection of an LD$_{50}$ dose of cocaine fails to alter time to peak plasma cocaine concentration or peak plasma concentration of cocaine (Sun et al., 2002), suggesting that this enzyme would fail to protect significantly against cocaine overdose.

Several mutants of BChe exhibiting greater catalytic efficiency than the native enzyme have been engineered. One mutant, A328Y, demonstrated an improved k_{cat} compared with BChe (k_{cat} = 10.2 min^{-1} and K_M = 9 μM) (Xie et al., 1999). Another mutant, A328W/Y332A has been reported to have an increase of approximately 9-fold in catalytic efficiency (k_{cat}/K_M) compared with the wild-type enzyme, with a k_{cat} of 154 min^{-1} and K_M of 18 μM. This enzyme significantly reduced cocaine-half-life and peak cocaine-plasma levels in rats (Sun et al., 2002) and decreased cocaine-induced locomotor activity by 80% in mice (Duysen et al., 2002). In addition, this mutant both blocked and reversed the hypertensive effects of cocaine in rats when given as a pre- or post-treatment, respectively (Gao and Brimijoin, 2004). Recent reports have demonstrated that a quadruple mutant, A199S/S287G/A328W/Y332G, derived from transition-state simulation, showed a 460-fold improvement in catalytic efficiency (Pan et al., 2005) compared with the wild-type enzyme.

Although some of these cocaine-hydrolyzing agents block behavioral effects of cocaine, they are weak in their capacity to prevent cocaine toxicity. To protect against toxicity, robust catalytic efficiency of the cocaine-hydrolyzing agent is required to protect against doses of cocaine that exceed the threshold LD100 when given both before and after cocaine administration.

A bacterium, *Rhodococcus* sp. MB1, indigenous to the soil surrounding the coca plant, has evolved the capacity to use cocaine as its sole carbon and nitrogen source. The bacterium expresses a cocaine esterase (CocE) (Bresler et al., 2000; Larsen et al., 2002; Turner et al., 2002) that acts just as Mab

15A10 and BChe to hydrolyze the benzoyl ester of cocaine (Fig. 1). The gene for CocE has been isolated and cloned (Bresler et al., 2000), and the crystal structure of CocE has been determined (Larsen et al., 2002; Turner et al., 2002). The structure of CocE (Fig. 4a) reveals a classic serine esterase fold in addition to two other domains that combine to form a cocaine binding pocket. Altering any of the amino acids (Asp, His, or Ser) of the catalytic triad (for review, see Dodson and Wlodawer, 1998) inactivates the esterase. Furthermore, mutation of residues that make contact with the benzoate moiety of cocaine (e.g., Tyr44) also disrupts cocaine hydrolysis, presumably through impairing oxyanion stabilization in the transition state (Larsen et al., 2002; Turner et al., 2002). The purified enzyme ($\sim$65 kDa) catalyzes cocaine hydrolysis (k_{cat} = 7.8 s^{-1} and K_M = 640 nM) (Larsen et al., 2002; Turner et al., 2002) with an efficiency nearly 3 orders of magnitude greater than endogenous esterases and, most likely, sufficient to detoxify a clinical overdose (Landry et al., 1993; Mets et al., 1998). In addition, the esterase also metabolizes cocaethylene, a potent metabolite of cocaine and alcohol, almost as efficiently as cocaine (k_{cat} = 9.4 s^{-1} and K_M = 1600 nM) (Larsen et al., 2002; Turner et al., 2002).

Given that CocE is a cytosolic protein derived from a bacterial source, it was unclear whether the enzyme would be sufficiently stable in vivo. The catalytic efficiency and stability of the esterase have been shown to be sensitive to changes in pH (Turner et al., 2002). To determine CocE's esteratic activity in vivo, a rodent model of acute cocaine toxicity was implemented. When treated with high doses of cocaine, rats first exhibit convulsions followed by cessation of respiration and movement. The lowest fatally toxic dose of cocaine (LD100), when administered i.p., will produce death within 15 min of treatment using our procedure. Protection against cocaine-induced lethality by CocE was determined and compared with the protective effects of human BChe. The essential esteratic activity of CocE was established by assessing the activity of two mutant enzymes, each lacking a key amino acid of the active site. In addition, activity of a modified wild-type enzyme by a covalent modification of serine by phenylmethylsulfonyl fluoride (PMSF) was determined. Esteratic degradation of cocaine was further proven to be the specific mechanism of CocE's protective effects by verifying inactivity against WIN-35065-2 (e.g., Madras et al., 1989), a cocaine analog that lacks the benzoyl ester targeted by CocE. Enzyme kinetics of CocE were determined in both human serum and rat plasma, providing a measure of both enzyme activity and half-life under isolated but physiological conditions. The in vitro catalytic half-life was then compared with its in vivo half-life by administering the enzyme at increasing periods before cocaine treatment.
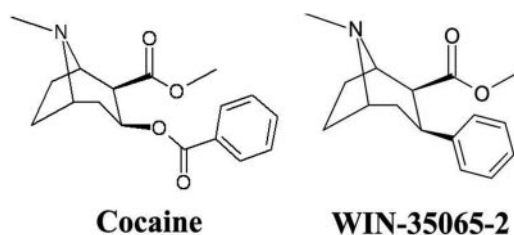

Fig. 1. Structures of cocaine and WIN-35065-2. BChe and CocE cleave cocaine at the benzoyl ester bond to produce two nonpsychoactive metabolites, ecgonine methyl ester and benzoic acid. WIN-35065-2 lacks this ester bond.

Materials and Methods

Drugs. Cocaine was obtained from the National Institute on Drug Abuse (Bethesda, MD). WIN-35065-2 was provided by Dr. Ivy Carroll (National Institute on Drug Abuse, Research Triangle Institute, NC). Both drugs were dissolved in sterile water. Purified human butyrylcholinesterase was a gift from Dr. Oksana Lockridge (University of Nebraska, Omaha, NE).

Mutagenesis. Standard desalted primers for mutagenesis were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). QuikChange mutagenesis was performed using the primers listed below and according to the manufacturer's specifications (Stratagene, La Jolla, CA). The codon that was mutated is underlined: Y44F, 5-tcgcaacccattcgacaagttcg-3′; S117A, 5′-gttcggcgttgcgtactt-gggtg-3′. Incorporation of the mutation was confirmed by DNA sequencing by the University of Michigan DNA sequencing core.

Toxicity Studies. Male Sprague-Dawley rats (300 g) were obtained from Harlan (Indianapolis, IN) and housed three animals per cage. After surgical implantation of a jugular catheter (see below), all rats were individually housed until the termination of the experiment. Rats were maintained on a 12-h light/dark cycle, with lights turned on at 7:30 AM, and food and water were available ad libitum. All studies were carried out in accordance with protocols approved by the University of Michigan University Committee on the Use and Care for Animals.

Surgery. Rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Intravenous catheters were made from Micro-Renathane tubing (15 cm; Braintree Scientific Inc., Braintree, MA) and were implanted in the right jugular vein. Approximately 3 cm of the catheter was inserted in the vein; the remaining tubing was passed subcutaneously to the back, where it exited from an incision made between the shoulder blades. The exposed tubing was capped with a 1-cm piece of stainless steel (0.28 diameter; Small Parts Inc., Miami, FL). Catheters were flushed daily with 0.5 ml of heparinized saline (50 U/ml) to maintain catheter patency. After surgery, rats were allowed 1 week to recover. Each rat was used for a single experiment, and all experimental groups consisted of six to eight rats.

Dosing Regimen. To determine the lowest effective dose of CocE that blocked cocaine-induced convulsions and death, 0.1, 0.32, or 1.0 mg of CocE or vehicle [phosphate-buffered saline (PBS)] was administered i.v. 1 min after 180 mg/kg cocaine (i.p.). To determine the catalytic limits of CocE, increasing doses of cocaine were administered (100, 560, 1000 mg/kg, i.p.) one min before 1.0 mg of CocE (i.v.). Mutants and PMSF-blocked CocE were administered (1 mg, i.v.) one min before 180 mg/kg cocaine (i.p.). CocE (1.0 mg, i.v.) was also administered one min after WIN-35065-2 (560 mg/kg, i.p.). CocE (1.0 mg, i.v.) was given before and after cocaine (100 mg/kg, i.p.) to determine the in vivo half-life of the esterase. All intravenous injections were followed by a heparinized saline flush (0.5 ml). After treatment, rats were observed for convulsions and death. Number of convulsant episodes, duration of each episode, and type of convulsion were recorded. Death was defined as cessation of observed movement and respiration.

Data Analysis. The number of rats exhibiting cocaine toxicity (death and convulsions) was calculated as a percentage for each treatment.

Determination of Enzyme Activity in Human Plasma. Discarded human plasma from the University of Michigan Hospital blood bank was used for the determination of cocaine levels after cocaine esterase treatment. Aliquots (3 ml) of human plasma were maintained at 37°C in a water bath for 10 min before the start and for the duration of the experiment. After equilibrating plasma in the water bath, cocaine was added to a final concentration of 300 μM and vortexed for 30 s. Plasma samples (20 μl) were removed and placed in a microcentrifuge tube containing the internal standard and a saturated sodium fluoride solution to prevent further cocaine metabolism. Immediately after taking the first plasma sample (cocaine alone), cocaine esterase or vehicle solution was added and vortexed. Plasma samples were collected 1, 2, 4, 6, 8, 10, 15, 30, 45, 60, and 120 min after adding the esterase.

Extractions. Compound extractions from human plasma samples were performed in 100% acetonitrile (3× volume), incubated for approximately 15 min, centrifuged at 13,000 rpm for 45 min, and the resulting supernatant was collected. The extracts were concentrated on a Speed Vac (Thermo Electron Corp., Waltham, MA) to remove the acetonitrile. Extracted samples were reconstituted in water and further diluted 10–1000 times.

Liquid Chromatography. The chromatography was performed using a Surveyor high-performance liquid chromatography system (Thermo Electron Corp.) with a quaternary pump and autosampler configured with a 10-μl injection loop. Separation was achieved using a Phenomenex C18 3 μm 30 × 4.6-mm column with corresponding guard column (Waters Corp., Milford, MA) at a flow rate of 600 μl/min. Solvent A consisted of a 0.1% formic acid solution, and solvent B was 0.1% formic acid in acetonitrile (high-purity grade; Honeywell Burdick & Jackson (Meskegon, MI). A 3-min ballistic gradient was used with cocaine, and the internal standard coeluted at 2.3 min.

Mass Spectrometry. For detection and quantification, a TSQ Quantum Ultra AM triple quadrupole mass spectrometer equipped with an IonMax electrospray ionization source (Thermo Electron Corp.) was used in positive ion, selected reaction monitoring mode. Nitrogen served as the nebulizing gas and argon as the collision gas. Gas flow rates, spray voltages, and collision energies were optimized. Calibration curves were determined for cocaine with 50 nM deuterated cocaine (cocaine D$_3$) as the internal standard in untreated plasma samples. Unknown samples were also spiked with cocaine D$_3$. All samples were evaluated in triplicate. Standard curves and unknowns were analyzed by Quan Browser program in Xcalibur version 1.4 (Thermo Electron Corp.) software. Calibration curves were constructed using linear regression of cocaine peak area/internal standard area ratio as a function of standard concentration with a weighting factor of 1/x. Standard curve-fit values were accepted at greater than 0.99, and relative S.D. values for replicate samples were between 0 and 10%.

Determination of Enzyme Activity in Rat Serum. Enzyme activity was measured using a spectrophotometric assay under similar conditions as described by Turner et al. (2002). In brief, preparations of purified CocE were incubated with varying concentrations of cocaine at 25 C°. The unique absorption spectra of cocaine (extinction coefficient, 6.7 L/mmol/cm, at 240 nm) allowed for the observation of remaining cocaine after enzymatic cleavage. The initial linear rates of decay of cocaine, representing the velocity, were determined on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA) using SOFTmax Pro software (version 1.13). The reaction was
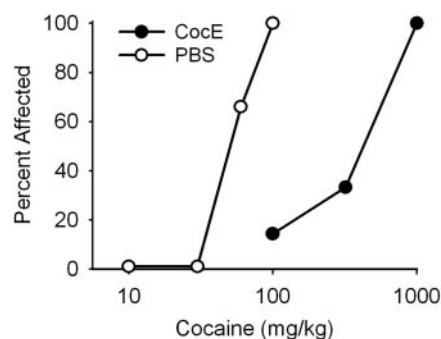

Fig. 2. Effect of 1.0 mg of CocE (●) or PBS (vehicle, ○) on cocaine-induced lethality when administered i.v. 1 min after increasing doses of cocaine, administered i.p. (10 mg/kg cocaine + PBS, $n = 6$; 30 mg/kg cocaine + PBS, $n = 6$; 60 mg/kg cocaine + PBS, $n = 6$; 100 mg/kg cocaine + PBS, $n = 6$; 100 mg/kg cocaine + CocE, $n = 7$; 320 mg/kg cocaine + CocE, $n = 6$; and 1000 mg/kg cocaine + CocE, $n = 6$). Data presented are expressed as a percentage of each group exhibiting cocaine-induced fatality.

initiated by adding 150 μL of a 2× enzyme solution to 150 μL of a 2× cocaine solution. Final CocE concentrations ranged from 100 to 20 ng/ml. Final cocaine concentrations were as follows: 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 μM. The buffer used was phosphate-buffered saline, pH 7.4. Initial rates were fit to the Michaelis-Menten equation, with k_{cat} and K_M as adjustable parameters (Prism version 4; GraphPad Software, San Diego, CA).

Results and Discussion

In the rodent model of acute toxicity, cocaine dose-dependently induced convulsions and death in rats; death was observed in less than 15 min after administration in 100% of animals given 100 mg/kg cocaine (Fig. 2). CocE (1.0 mg) infused after cocaine administration produced a 10-fold shift
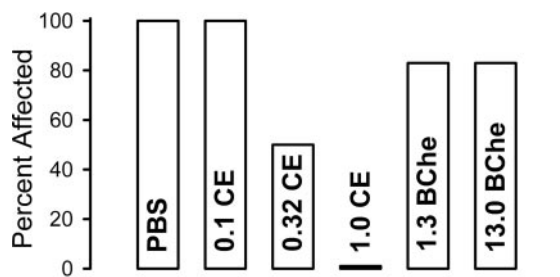

Fig. 3. Increasing doses of CocE (CE), human BChe (BChe), or PBS were administered i.v. 1 min before 180 mg/kg cocaine (i.p.), and subjects were observed for fatality (PBS, $n = 6$; 0.1 mg of CocE, $n = 7$; 0.32 mg of CocE, $n = 6$; 1.0 mg of CocE, $n = 6$; 1.3 mg of BChe, $n = 6$; 13.0 mg of BChe, $n = 6$). Data presented are expressed as a percentage of each group exhibiting cocaine-induced fatality.
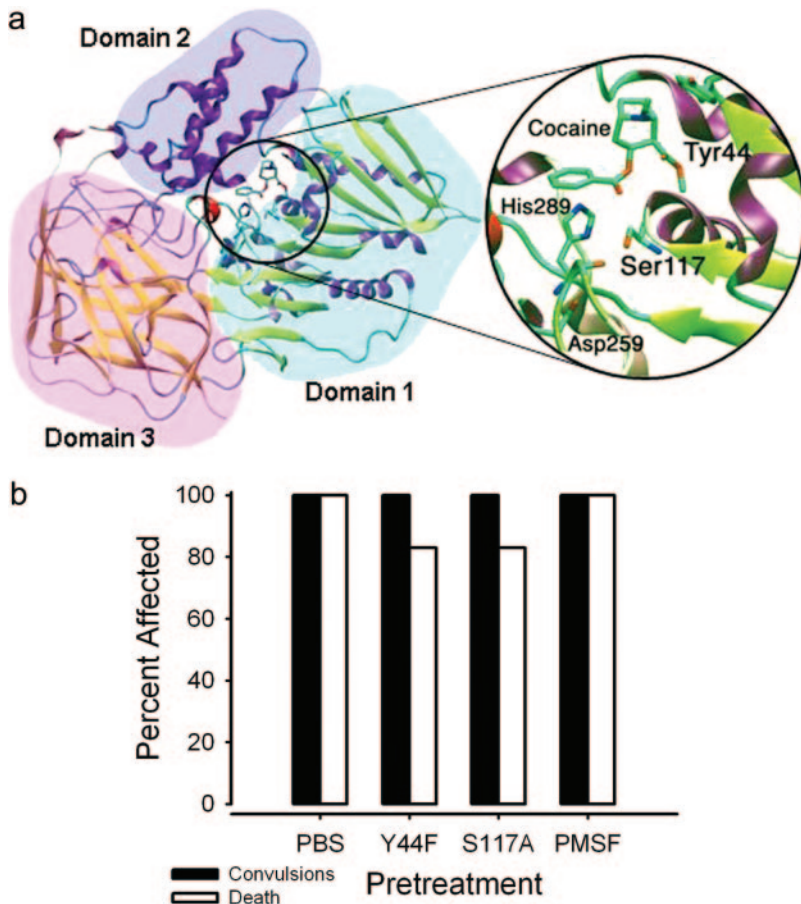
in the cocaine-toxicity dose effect curve (Fig. 2): 1000 mg/kg cocaine was required to surmount the protective, catalytic properties of CocE. No other reported esterase has been able to shift the dose-effect curve for cocaine-induced lethality to this extent. Furthermore, this protocol closely resembles human toxicity situations, where the antidote is given only after cocaine has been ingested, inhaled, or injected. The superior catalytic efficiency of CocE compared with human BChe in this paradigm is substantial. Given 1 min before 180 mg/kg cocaine, 1 mg of CocE offered 100% protection against cocaine-induced lethality (Fig. 3), whereas a 10-fold molar equivalent dose of human BChe (13 mg) offered no protection (Fig. 3).

Both inactivating mutations of CocE (Ser117Ala or Tyr44PhE) lacked in vivo activity and, therefore, had no protective effects (Fig. 4b). Furthermore, PMSF treatment also eliminated CocE's protective effect against cocaine (Fig. 4b). In addition, the lethal effect of the nonhydrolyzable cocaine analog WIN-35065-2 was not diminished by treatment with CocE (Fig. 5). Based upon in vivo protection studies performed with catalytically inactivated preparations of the enzyme (PMSF-treated and CocE mutants), it is clear that the protective effects of the enzyme are due to its ability to hydrolyze cocaine. Taken together, these data are consistent with in vitro assessments of CocE's esteratic activity (Turner et al., 2002) and confirm the enzyme's mechanism of protection against cocaine-induced lethality in vivo.

CocE was found to have time-dependent protective effects; 100% of rats were saved when treated with CocE (1 mg) 1 min before cocaine, whereas only 66.7% and 33.3% of rats

Fig. 4. *a*, ribbon diagram of CocE highlighting its active site. The Asp259, His289, and Ser117 residues contribute to the cocaine-hydrolyzing properties of the enzyme, whereas the Tyr44 residue allows the enzyme to make contact with the benzoate moiety of cocaine. *b*, CocE mutants S117A, Y44F, or PMSF-treated CocE was administered i.v. 1 min before treatment of 180 mg/kg cocaine (i.p.) (PBS, $n = 6$; S117A, $n = 6$; Y44F, $n = 6$; PMSF, $n = 5$). Data presented demonstrate percentage of each group exhibiting convulsions (black bars) and death (open bars).

survived when treated with CocE 10 and 30 min before cocaine, respectively (Fig. 6b). CocE's protective effects were eliminated when rats were treated 100 min before cocaine. This time-dependent effect is probably due to the thermal deactivation of the enzyme in vivo. In rat plasma, CocE was
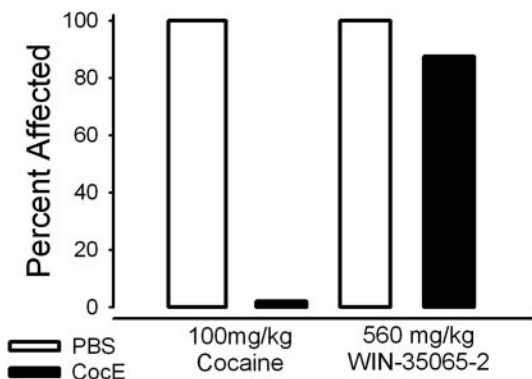

Fig. 5. CocE (1.0 mg, i.v.) or PBS (i.v.) was administered 1 min before 100 mg/kg cocaine (i.p., PBS + cocaine, $n = 6$: CocE + cocaine, $n = 6$) or the determined LD100 of the cocaine-analog WIN-35065-2 (560 mg/kg, i.p., PBS + WIN-354065–2, $n = 8$; CocE + WIN-35065-2, $n = 7$). Data presented demonstrate percentage of each group exhibiting death after pretreatment with PBS (open bars) or CocE (black bars).
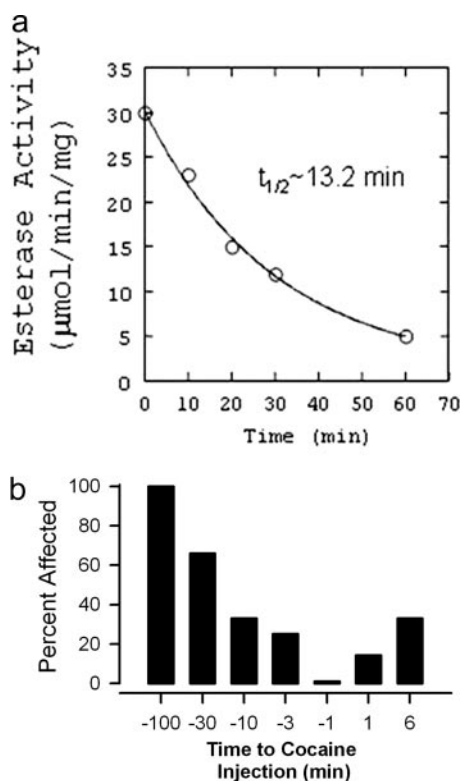

Fig. 6. *a*, time-dependent inactivation of CocE in vitro. Purified CocE enzyme (at 250 ng/ml) was incubated in assay buffer in the absence of cocaine at 37°C for various times. After incubation, the samples were placed on ice and samples were then incubated with cocaine. The rate of decay of cocaine at A240 was measured on a multiplate reader as described under *Materials and Methods* to derive k_{cat} for CocE at each time point. The data were fitted to a single exponential decay using Kaleidagraph (Abelbeck/Synergy, Reading, PA), yielding a $t_{1/2}$ of 13.2. *b*, CocE (1 mg, i.v.) was administered at varying times before and after 100 mg/kg cocaine (i.p.) to determine the time-dependent protective effects the enzyme. Data presented demonstrate percent of each group exhibiting death after cocaine treatment (−100 min, $n = 6$; −30 min, $n = 6$; −10 min, $n = 6$; −3 min, $n = 8$; −1 min, $n = 8$; 1 min, $n = 7$; 6 min, $n = 6$).

found to have a half-life of 13 min (Fig. 6a), probably due to sensitivity to changes in pH and temperature. Given these data, it can be approximated that a 1-mg dose of CocE administered 30 min before cocaine will decay approximately 2 half-lives, leaving 0.25 mg of enzyme in the general circulation when cocaine is administered. Because of its high catalytic efficiency, this small concentration affords some protection, as we observe that a third of animals were protected from toxicity under these conditions (Fig. 6b). These data are in agreement with dose-dependent protective effects of CocE when given 1 min before cocaine (Fig. 3).

We are, of course, interested in whether CocE's protective effects might work in humans. We assume that the enzyme distributes quickly and evenly in the blood. Previous studies have suggested that lethal blood concentrations of cocaine in the rat vary between 50–128 μM (Mets and Virag, 1995; Mets et al., 1999), and peak plasma levels of cocaine occur approximately 13 min after an i.p. injection (Sun et al., 2002). Based on reported kinetics of i.p. cocaine administration (Sun et al., 2002), it is estimated that 100 and 320 mg/kg cocaine yield peak cocaine blood concentration of 35 and 113 μM, respectively. Lethal concentrations of cocaine are of a similar magnitude in humans (3–200 μM) (Finkle and McCloskey, 1978; Wetli and Wright, 1979; Baselt, 2002), with the exception of one report citing a fatality by oral overdose, in which the decedent was found to have cocaine blood levels of 695 μM due to massive oral cocaine ingestion (Amon et al., 1986). Because 1 mg of CocE saved rats treated with these doses of cocaine (Fig. 2), it can be justifiably predicted that the enzyme might protect against cocaine toxicity in humans. Furthermore, in human plasma spiked with 300 μM cocaine, a concentration that exceeds the average reported toxic levels of cocaine, and then treated with CocE (a molar equivalent of our in vivo 1.0-mg dose), the cocaine concentration was reduced to approximately 2 μM in less than a min (Fig. 7). Thus, we have extended the finding from the rodent studies, and demonstrated CocE's rapid hydrolytic activity in human plasma.

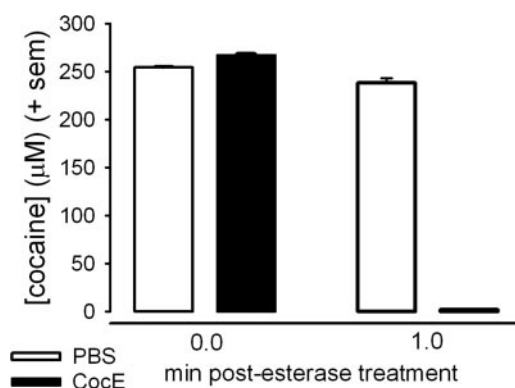In conscious rats, 10 mg/kg BChe (i.v.) delivered 3 min after the LD_{50} dose of cocaine (80 mg/kg, i.p.) protected

Fig. 7. Cocaine concentrations in human plasma treated with 0.8 μM cocaine esterase or esterase vehicle. Human plasma samples were spiked with 300 μM cocaine and maintained at 37°C. One aliquot of plasma was sampled before the addition of cocaine esterase or esterase vehicle, and another aliquot was collected 1 min after esterase addition. Plasma aliquots were mixed immediately with the internal standard (cocaine-D_3) and a saturated sodium fluoride solution to prevent further cocaine metabolism. Levels of cocaine and internal standard were quantified by HPLC with tandem mass spectrometry.

against cocaine-induced lethality (Lynch et al., 1997). Assuming that the enzyme is distributed similarly in the human, a 70-kg individual would require a 700-mg dose of exogenous BChe to protect against an overdose. In cats, BChe administration (0.27 mg/kg, i.v.) attenuated cocaine-induced widening of the QRS complex when given 1 min after cocaine (3 mg/kg i.v., at a rate of 1.2 ml/min) from 59 ± 9 to 50 ± 5 ms and blocked cocaine-induced hypertension (1 mg/kg, i.v.) when given as a 2-min pretreatment (Mattes et al., 1997). In anesthetized rats, a pretreatment of BChe (7.8 mg/kg, i.v.) increased the dose of cocaine required to produce cardiovascular collapse from 1.0 to 8.72 mg/kg (i.v.). Although BChe has been shown to protect against some of cocaine's toxic actions when administered before or after cocaine, there is no evidence that the enzyme can reverse cocaine toxicity when administered after a dose equivalent to or exceeding cocaine's LD100. We have demonstrated that a dose of 1 mg of CocE in a 300-g rat is sufficient to protect against a dose of cocaine that exceeds the LD100 (Fig. 2). In addition, the enzyme given both before and, more importantly, up to 6 min after the LD100 dose of cocaine provided protection from toxicity (Fig. 6b). This dose of cocaine induced convulsions 6 min after its administration, followed by little or no locomotor activity, accompanied by labored breathing, until cessation of movement and breathing was observed 10 to 15 min after the cocaine treatment. CocE's ability to protect against fatality when administered 6 min after cocaine provides proof of concept that the enzyme can prevent cocaine-induced lethality when administered at a time-point proximate to death. Administration of CocE after the observed convulsion would further strengthen the implications of CocE's clinical application. In human plasma, CocE metabolized cocaine concentrations by 150-fold in less than one min (Fig. 7). Given these data, we predict that 250 mg of CocE administered to a 70-kg human after toxic cocaine ingestion would rescue the individual.

Although CocE is hypothesized to reverse the direct lethal effects of cocaine in humans, it is unlikely that enzyme administration would reverse other toxic effects, such as damage to the myocardium, a concern when considering CocE as a potential antidote to cocaine toxicity. Furthermore, because CocE is a protein that is foreign to the mammalian genome, immunogenicity is an issue that must be addressed. Preliminary studies with Rhesus monkeys have demonstrated minimal immunogenicity with repeated administration of 1.8 mg/kg CocE i.v. CocE's hydrolytic activity of cocaine is unchanged, and anaphylaxis has not been observed (M. C. Ko, E. M. Jutkiewicz, and J. H. Woods, unpublished results). Nevertheless, a positive attribute of being bacterially derived is that production of CocE is more time- and cost-efficient compared with BChe production, because of the rapid growth of bacteria and consequent protein expression. BChe is derived from mammalian cells, which have a characteristically slow growth rate and poor protein expression. More effective methods for BChe production, however, have been described in transgenic goats (Cerasoli et al., 2005).

The in vivo assessments reported herein clearly indicate that i.v. administration of purified CocE is capable of reversing the lethal effects of high doses of cocaine in the rat. The potency and effectiveness of CocE treatment of cocaine toxicity in rats suggests that administration of CocE as treatment for cocaine overdose in humans is plausible. This effective antidote for cocaine lethality would represent the first major advance in the acute treatment of clinical cocaine and crack-cocaine overdoses observed in hospital emergency rooms. Moreover, if CocE's thermostability can be extended and its immunogenicity blocked [e.g., through pegylation (Harris and Chess, 2003)], CocE may become a useful pharmacotherapy for cocaine abuse.

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