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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, BChe, 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.

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INTRODUCTION

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 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 anti-cocaine catalytic antibody (Landry *et al*., 1993; Carroll *et al*., 1999; Larsen *et al*., 2004) or an endogenous esterase such as butyrylcholinesterase (BChe).

The anticocaine catalytic antibody, mAb 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

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metabolites are non-toxic in mammals. mAb 15A10 is reported to decrease intravenous cocaine self-administration while not suppressing food-maintained responding in rats (Baird *et al*., 2000). Additionally, 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 at extremely high doses of the antibody (15-50 mg/kg) due to 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 (Mattes *et al*., 1997; Lynch *et al*., 1997; Carmona *et a*l., 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 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 hours), probably because it is produced endogenously and is, therefore, stable under physiologic conditions (see Gorelick, 1997, for review). Despite the catalytic properties and long half-life of BChe, the enzyme is not necessarily a viable therapeutic candidate for cocaine toxicity

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primarily due to inadequate catalytic efficiency (k_{cat} = 3.9 min^{-1} and K_M = 14 μM) (Xie *et al*., 1999). Pretreatment with BChe ten minutes before an intravenous or intraperitoneal injection of a LD50 dose of cocaine fails to alter time to peak plasma cocaine concentration or peak plasma concentration of cocaine (Sun *et al*., 2002), suggesting 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 to 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 about a 9-fold increase in catalytic efficiency (k_{cat} /K_M) compared to 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). Additionally, 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 to 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 in order to

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protect against doses of cocaine that exceed the threshold LD100 when given both before or after cocaine administration.

A bacterium, *Rhodococcus sp*. MB1, indigenous to the soil surrounding the coca plant, has evolved the capacity to utilize cocaine as its sole carbon and nitrogen source. The bacterium expresses a cocaine esterase (CocE) (Bresler *et al*., 2000; Turner *et al*., 2002; Larsen *et al*., 2002) that acts just as Mab 15A10 and BChe to hydrolyze the benzoyl ester of cocaine (Figure 1). The gene for CocE has been isolated and cloned (Bresler *et al*., 2000), and the crystal structure of CocE has been determined (Turner *et al*., 2002; Larsen *et al*., 2002). The structure of CocE (Figure 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 (Turner *et al*., 2002; Larsen *et al*., 2002). The purified enzyme (MW ~65 kDa) catalyzes cocaine hydrolysis (k_{cat} =7.8 s^{-1} and K_M = 640 nM) (Turner *et al*., 2002; Larsen *et al*., 2002) with an efficiency nearly three orders of magnitude greater than endogenous esterases and, most likely, sufficient to detoxify a clinical overdose (Landry *et al*., 1993; Mets *et al*., 1998). Additionally, 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) (Turner *et al*., 2002; Larsen *et al*., 2002).

Given that CocE is a cytosolic protein derived from a bacterial source, it was uncertain as to 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 intraperitoneally, will produce death within 15 minutes of treatment using our procedure. Protection against cocaine-induced lethality by CocE was determined and compared to 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. Additionally, activity of a modified wild-type enzyme by a covalent modification of serine by phenylmethyl sulphonate 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 which 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 to its *in vivo* half-life by administering the enzyme at increasing periods prior to cocaine treatment.

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MATERIALS AND METHODS

Drugs. Cocaine was obtained from The National Institute on Drug Abuse (Bethesda, MD, USA). WIN-35065-2, ((-)-3β-phenyltropane-2β-carboxylic acid methyl ester tartrate) was provided by Dr. Ivy Carroll (NIDA, Research Triangle Institute, NC, USA). 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. QuickChange™ (Invitrogen) mutagenesis was performed using the primers listed below and according to the manufacturer's specifications. Bold font corresponds to the codon that was mutated (Y44F) 5-tcgcaaccca**ttc**gacaagttcg-3', (S117A) 5'-gttcggcgtt**gcg**tacttgggtg-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 grams) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed 3 animals per cage. Following 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 a.m. 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.

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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, MRE-040, 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. Following surgery, rats were allowed one week to recover. Each rat was used for a single experiment, and all experimental groups consisted of 6-8 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 CocE or vehicle (phosphate buffered saline, PBS) was administered intravenously one minute 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 minute prior to 1.0 mg CocE (i.v.). Mutants and PMSF-blocked CocE were administered (1 mg, i.v.) one minute before 180 mg/kg cocaine (i.p.). CocE (1.0 mg, i.v.) was also administered one minute 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 following cocaine esterase treatment. Aliquots (3 ml) of human plasma were maintained at 37ºC in a water bath for 10 min prior to 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% acetronitrile (3x 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 Savant Speed Vac (ThermoElectron Corp., Franklin, MA) to remove the acetonitrile. Extracted samples were reconstituted in water and further diluted 10-1000 times.

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Liquid Chromatography. The chromatography was performed using a Surveyor HPLC system (ThermoElectron Corp., Franklin, MA) with a quaternary pump and autosampler configured with a 10 µl injection loop. Separation was achieved using a Phenomenex C18 3µm 30 x 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 acetronitrile (high purity grade; Burdick and Jackson, Muskegon, MI). A 3 min ballistic gradient was used with cocaine and the internal standard co-eluting at 2.3 min.

Mass Spectrometry. For detection and quantification, a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer equipped with an IonMax electrospray ionization source (ThermoElectron Corp., Franklin, MA) 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 (ThermoElectron Corp., Franklin, MA) 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 a value is greater than 0.99, and RSD values for replicate samples are between 0-10%.

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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). Briefly, 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 following enzymatic cleavage. The initial linear rates of decay of cocaine, representing the velocity, were determined on a SpectraMax 190 plate reader (Molecular Devices) using SOFTmax Pro software (Version 1.13). The reaction was initiated by adding 150 μL of a 2x enzyme solution to 150 μL of a 2x cocaine solution. Final CocE concentrations ranged from 100 ng/mL- 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 (GraphPad; PRISM, version 4).

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 minutes after administration in 100% of animals given 100 mg/kg cocaine (Figure 2). CocE (1.0 mg) infused after cocaine administration produced a ten-fold shift in the cocaine-toxicity dose effect curve (Figure 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

to human BChe in this paradigm is substantial. Given one minute before 180 mg/kg cocaine, 1 mg CocE offered 100% protection against cocaine-induced lethality (Figure 3), while a 10-fold molar equivalent dose of human BChe (13 mg) offered no protection (Figure 3).

Both inactivating mutations of CocE (Ser117Ala or Tyr44PhE) lacked *in vivo* activity and, therefore, had no protective effects (Figure 4b). Furthermore, PMSF treatment also eliminated CocE's protective effect against cocaine (Figure 4b). Additionally, the lethal effect of the nonhydrolyzable cocaine analog, WIN-35065-2, was not diminished by treatment with CocE (Figure 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 minute before cocaine, while only 66.7% and 33.3% of rats survived when treated with CocE 10 and 30 minutes before cocaine, respectively (Figure 6b). CocE's protective effects were eliminated when rats were treated 100 min prior to cocaine. This time-dependent effect is most likely due to the thermal deactivation of the enzyme *in vivo*. In rat plasma, CocE was found to have a half-life of 13 minutes (Figure 6a), most likely 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

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minutes prior to cocaine, will decay approximately 2 half-lives, leaving 0.25 mg of enzyme in the general circulation when cocaine is administered. Due to its high catalytic efficiency, this small concentration affords some protection, as we observe that 1/3 of animals were protected from toxicity under these conditions (Figure 6b). These data are in agreement with dose-dependent protective effects of CocE when given 1 minute before cocaine (Figure 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 about 13 minutes after an intraperitoneal injection (Sun *et al*., 2002). Based on reported kinetics of intraperitoneal cocaine administration (Sun *et al*., 2002), it is estimated that 100 and 320 mg/kg cocaine yield peak cocaine blood concentration of 35 μM 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 that 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 (Figure 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), reduced the cocaine concentration to approximately 2 μM in less than a minute (Figure

7). Thus, extending the finding from the rodent studies, demonstrating CocE's rapid hydrolytic activity in human plasma.

In conscious rats, 10 mg/kg BChe (i.v.) delivered 3 minutes after the LD50 dose of cocaine (80 mg/kg, i.p.) protected 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 minute after cocaine (3 mg/kg i.v., at a rate of 1.2 ml/min) from 59 ± 9 msec to 50 ± 5 msec, and blocked cocaine-induced hypertension (1 mg/kg, i.v.) when given as a 2 min pretreatment (Mattes *et al*., 1997). In anaesthetized rats, a pretreatment of BChe (7.8 mg/kg, i.v.) increased the dose of cocaine required to produce cardiovascular collapse from 1.0 mg/kg (i.v.) 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 CocE in a 300 gram rat is sufficient to protect against a dose of cocaine that exceeds the LD100 (Figure 2). Additionally, the enzyme given both before, and more importantly, up to 6 minutes after the LD100 dose of cocaine, provided protection from toxicity (Figure 6b). This dose of cocaine induced convulsions 6 minutes after its administration, followed by little or no locomotor activity, accompanied by labored breathing, until cessation of movement and breathing was observed 10-15 minutes after the cocaine treatment. CocE's ability to protect against fatality when administered 6 minutes 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 minute (Figure 7). Given these data, we predict that a 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 (Ko, Jutkiewicz, and 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, due to 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 have, however, 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

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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 tenable. 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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Footnotes

a) This research was supported by USPHS Grant DA021416-01.

b) Request reprints from Dr. James Woods, Department of Pharmacology, University of Michigan, 1301 MSRB III, 1150 West Med Cntr Dr., Ann Arbor, MI 48109-0632, USA (jhwoods@umich.edu)

c) [1] now at the Institute of Psychiatry and Neurology, Department of Pharmacology, Sobieskiego St. 9, 02-957, Warsaw, Poland

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FIGURE LEGENDS

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

Figure 2. Effect of 1.0 mg CocE (closed circles) or PBS (vehicle, open circles) on cocaine-induced lethality when administered intravenously one minute after increasing doses of cocaine, administered intraperitoneally (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; 1000 mg/kg cocaine + CocE, n = 6). Data presented are expressed as percent of each group exhibiting cocaine-induced fatality.

Figure 3. Increasing doses of CocE (CE), human BChe (BChe), or PBS were administered intravenously one minute before 180 mg/kg cocaine (i.p.), and subjects were observed for fatality (PBS, n = 6; 0.1 mg CocE, n = 7 ; 0.32 mg CocE, n = 6; 1.0 mg CocE, n = 6; 1.3 mg BChe, n = 6; 13.0 mg BChe mg, n = 6). Data presented are expressed as percent of each group exhibiting cocaine-induced fatality.

Figure 4a. Ribbon diagram of CocE highlighting its active site. The Asp259, His289, and Ser117 residues contribute to the cocaine-hydrolyzing properties of the enzyme, while the Tyr44 residue allows for the enzyme to make contact with the benzoate moiety of cocaine.

Figure 4b. CocE mutants S117A, Y44F, or PMSF-treated CocE was administered intravenously one minute prior to treatment of 180 mg/kg cocaine (i.p.) (PBS, n = 6;

S117A, n = 6; Y44F, n = 6; PMSF, n = 5). Data presented demonstrate percent of each group exhibiting convulsions (black bars) and death (open bars).

Figure 5. CocE (1.0 mg, i.v.) or PBS (i.v.) was administered one minute 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 percent of each group exhibiting death after pretreatment of PBS (open bars) or CocE (black bars).

Figure 6a. 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. Following 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 in the methods section to derive k_{cat} for CocE at each time point. The data were fitted to a single exponential decay using Kaleidagraph™ (Synergy software) yielding a $t_{1/2}$ of 13.2.

Figure 6b. CocE (1 mg, i.v.) was administered at varying times before and after 100 mg/kg cocaine (i.p.) in order 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).

Figure 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

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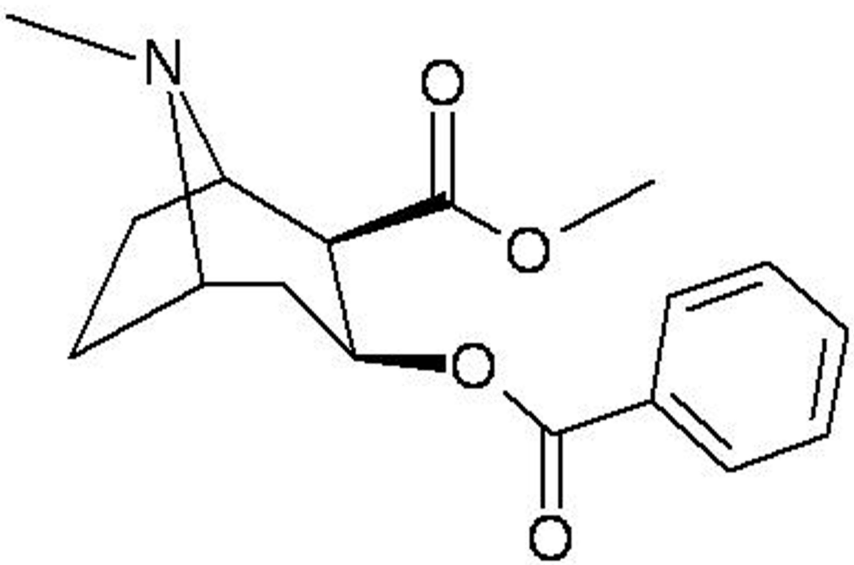
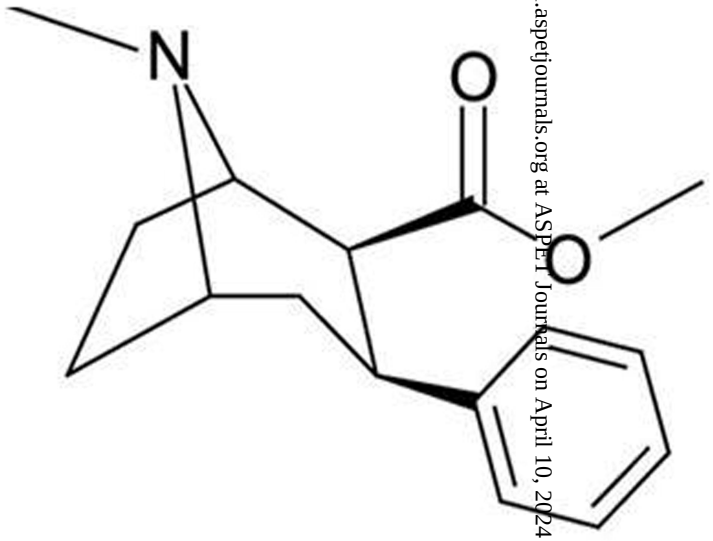
maintained at 37ºC. One aliquot of plasma was sampled prior to the addition of cocaine esterase or esterase vehicle, and another aliquot was collected 1 min following esterase addition. Plasma aliquots were mixed immediately with the internal standard (cocaine-D_3) and a saturated sodium fluoride solution to prevent further cocaine metabolism. Levels of cocaine and internal standard were quantified by HPLC with tandem mass spectrometry.

Figure 1



Cocaine



WIN-35065-2

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Figure 2

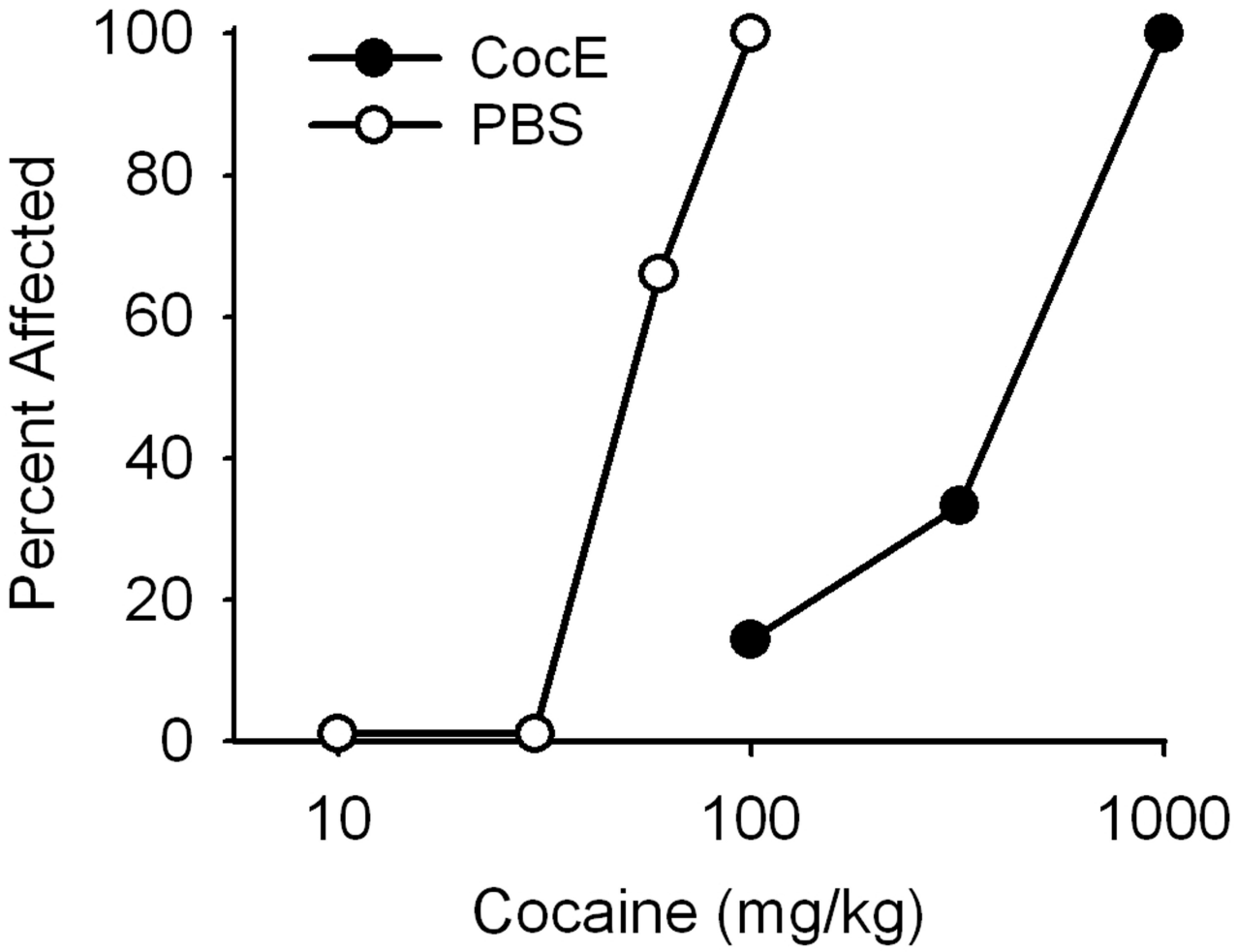

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Figure 3

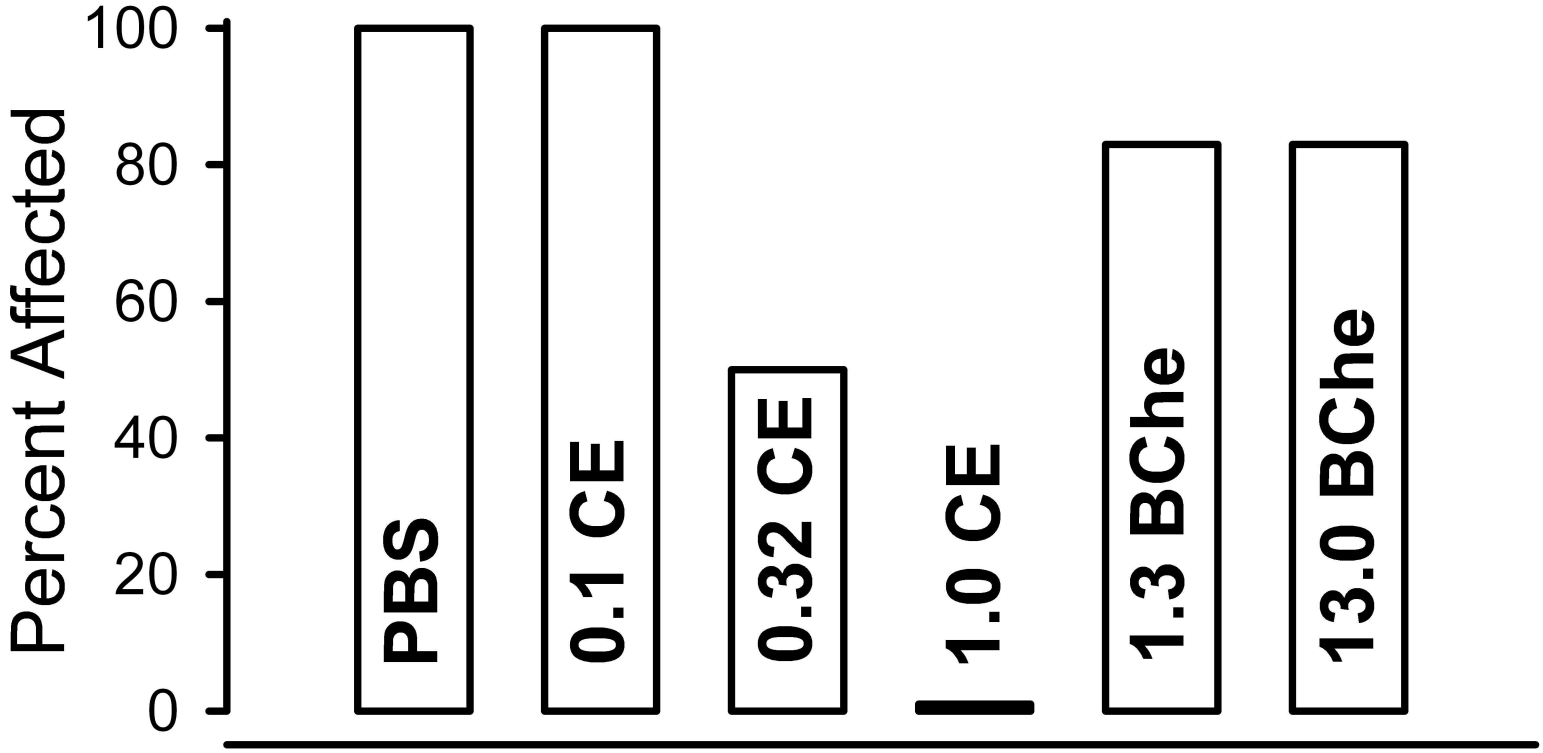

Figure 4a

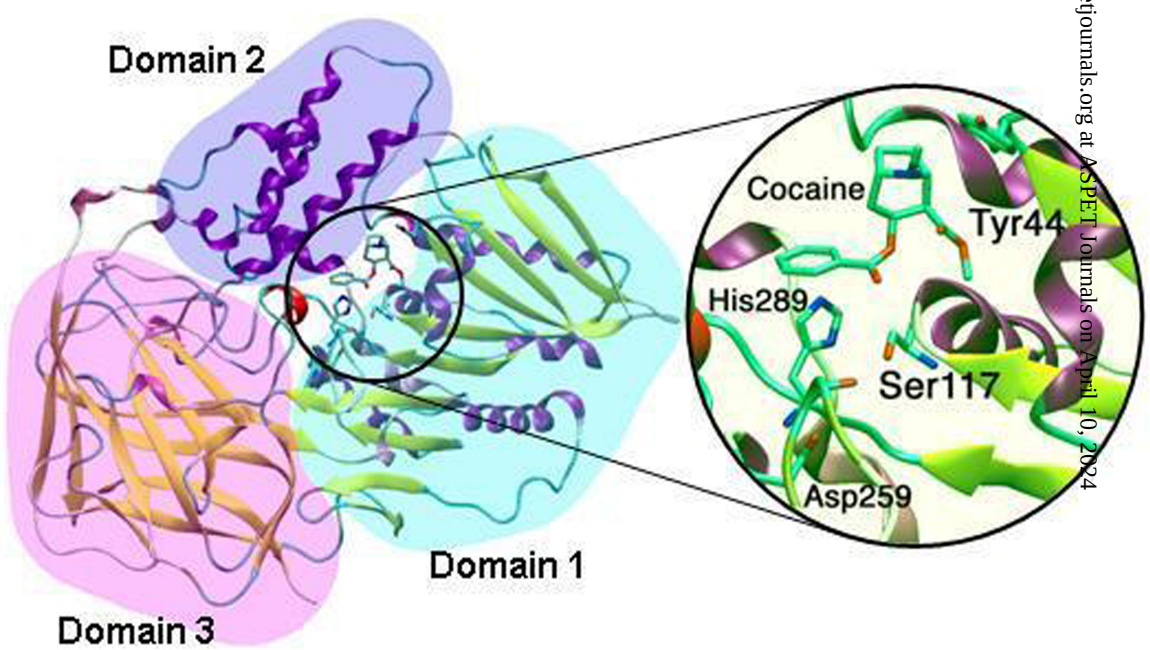

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Figure 4b

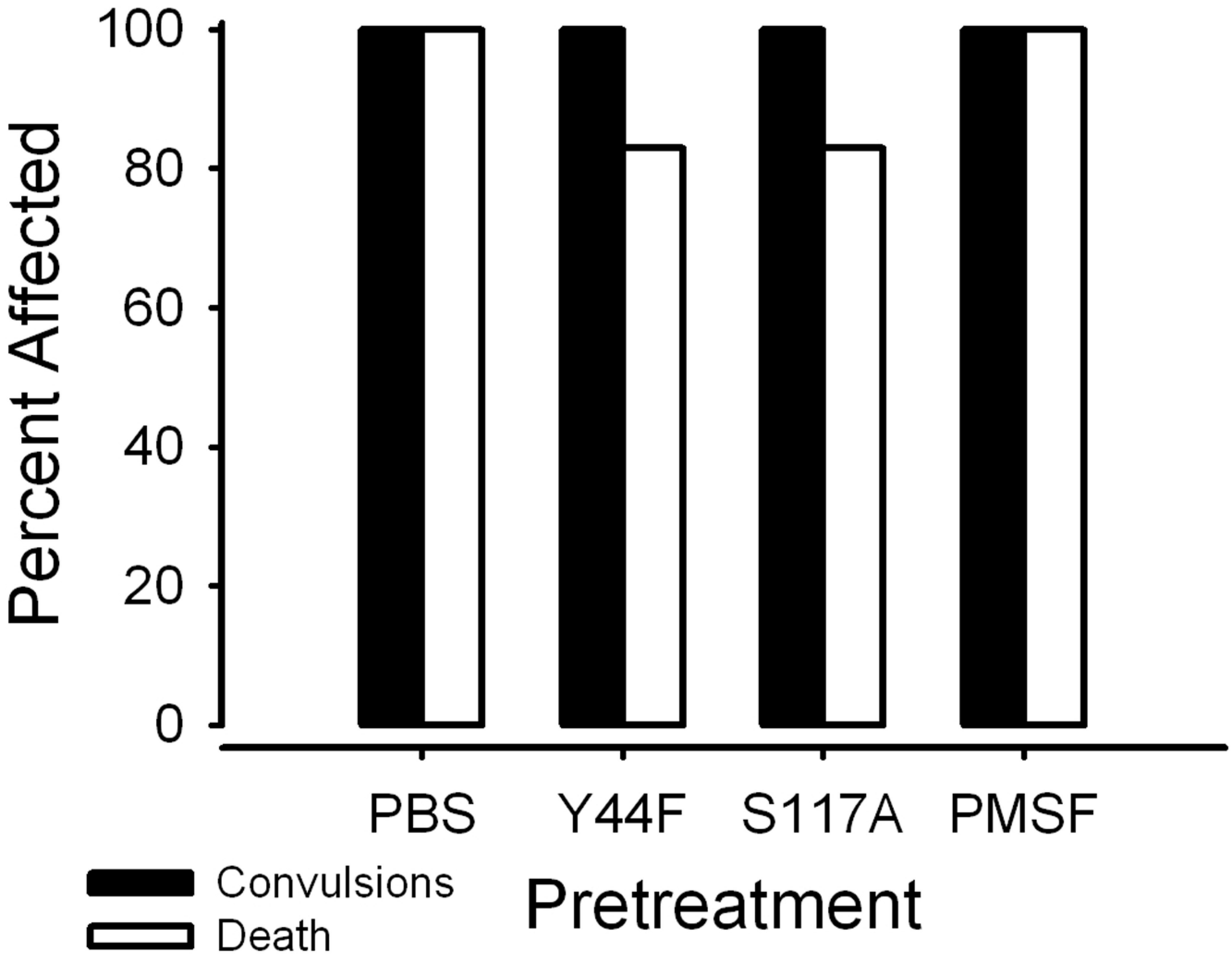

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Figure 5

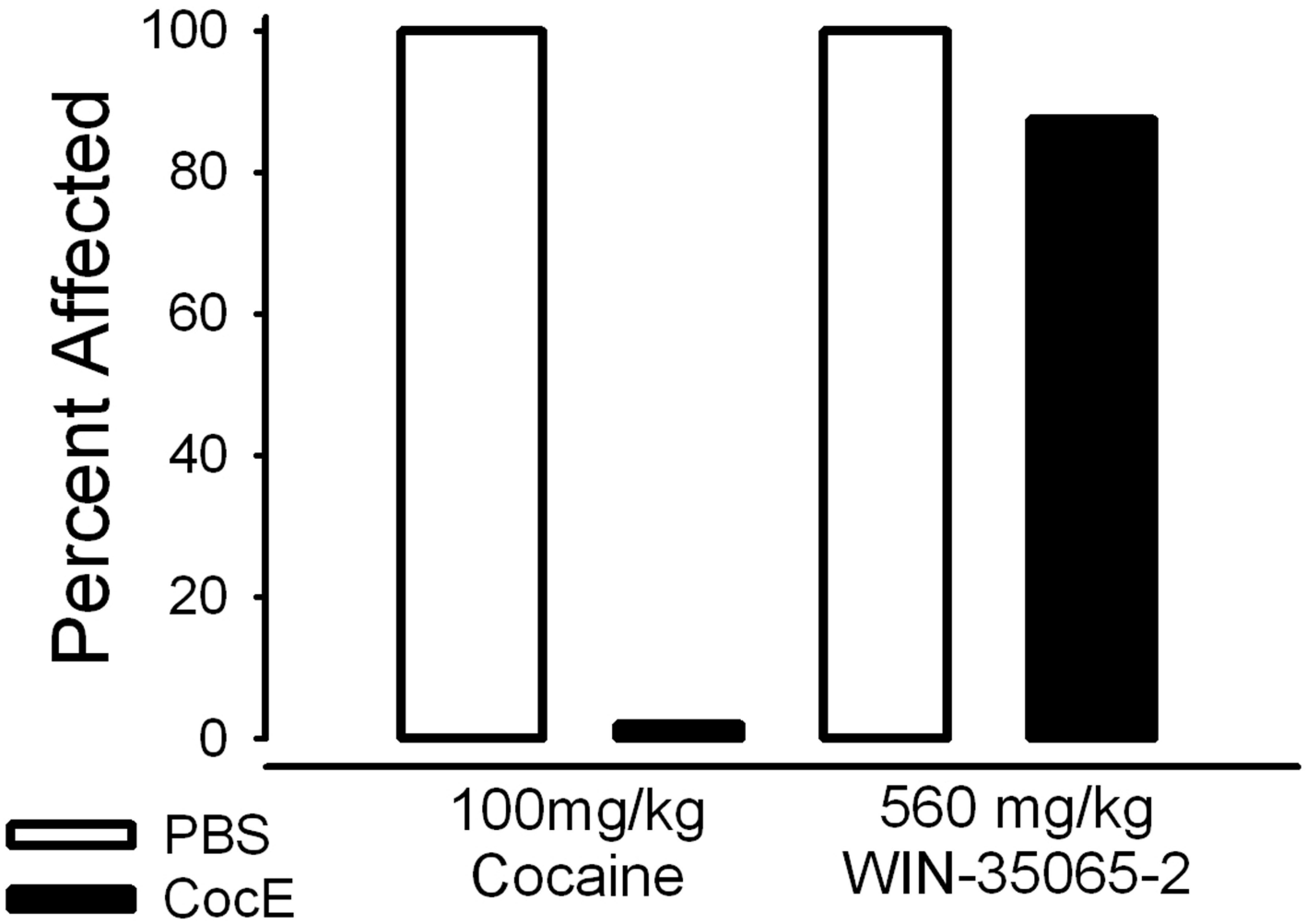

Figure 6a

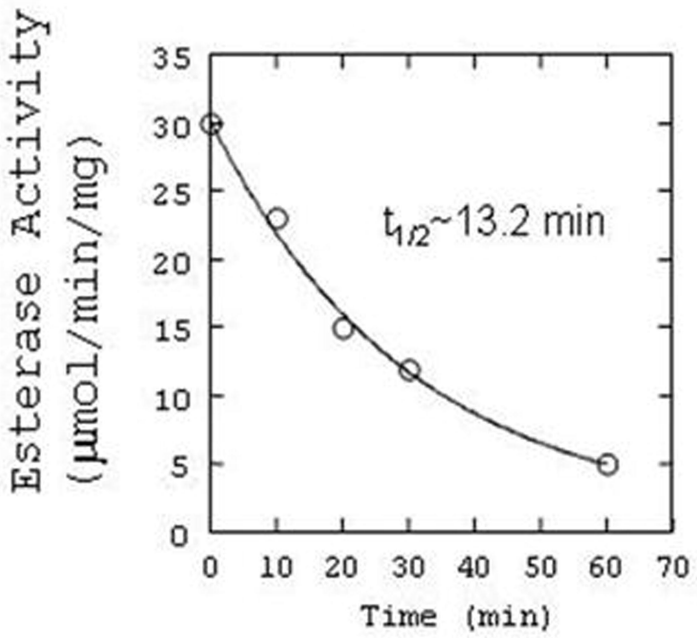

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Figure 6b

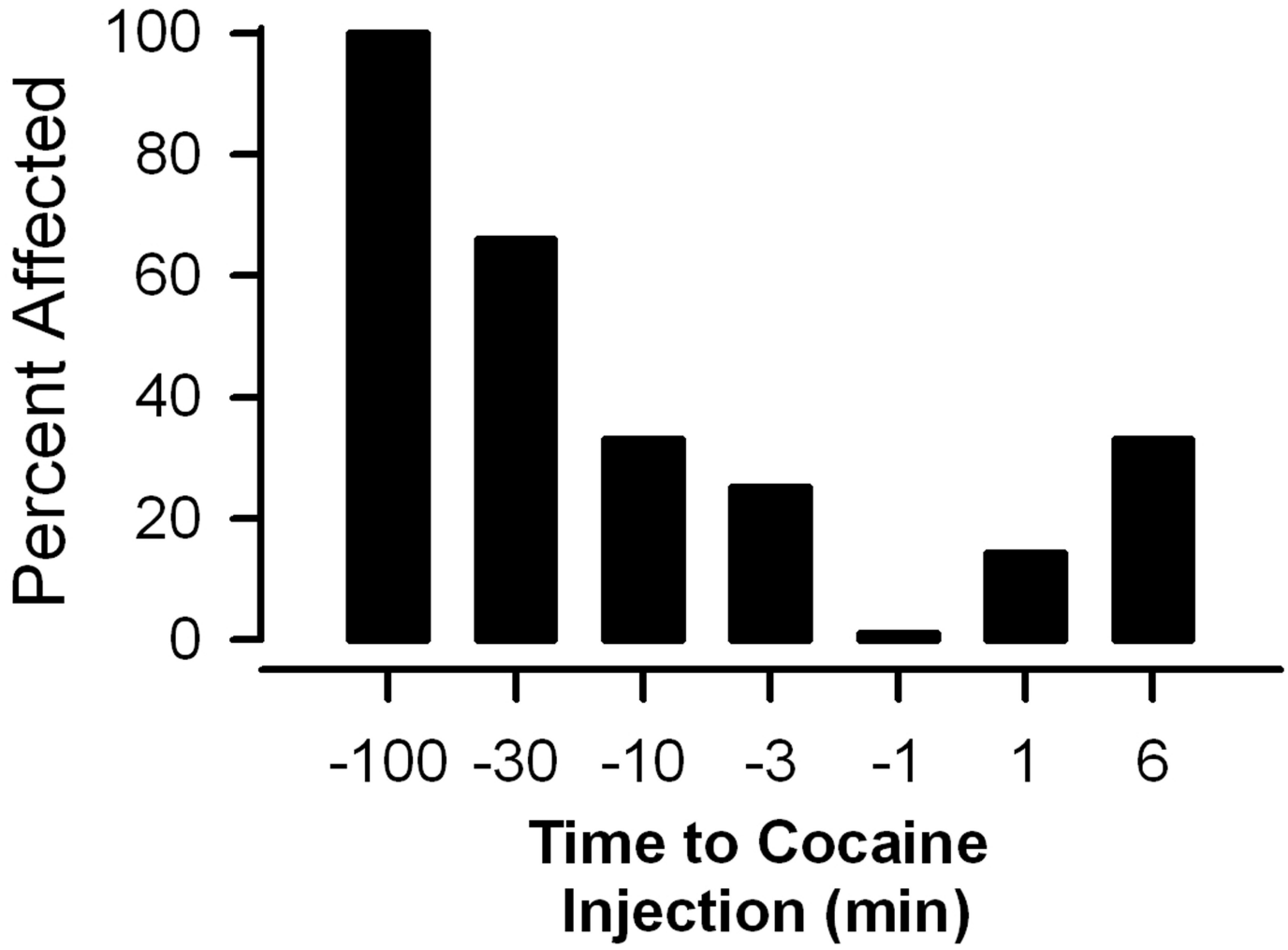

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Figure 7