

An Engineered Cocaine Hydrolase Blunts and Reverses Cardiovascular Responses to Cocaine in Rats

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ABSTRACT

There is increasing evidence that human plasma butyrylcholinesterase can lower the toxicity of cocaine overdose. Recently, with structure-based protein engineering, we converted this enzyme into a more efficient cocaine hydrolase (CocE). When tested in rats, CocE shortened cocaine's plasma half-life and decreased drug accumulation in heart and brain. Here, we have investigated the potential of CocE to antagonize cardiovascular responses to cocaine. Anesthetized rats were instrumented for continuous recording of blood pressure from the femoral artery. Cocaine (7 mg/kg i.v.) caused blood pressure to rise within 30 s by 25 to 37 mm Hg, but pressure returned to

baseline within 60 s. These transient pressor responses were prolonged up to 5 min when vagal reflexes were blocked with atropine (1 mg/kg). Under such conditions, pretreatment with CocE (3 mg/kg i.v.) reduced cocaine's pressor effect, whereas delayed treatment with CocE rapidly restored normal mean blood pressure. CocE had no hemodynamic effects in control animals not treated with cocaine. The finding that CocE can oppose pre-established physiologic actions of cocaine suggests that similar or improved hydrolases might help rescue patients from the life-threatening toxicity of drug overdose.

Because classic pharmacological treatments for cocaine overdose are poor, and effective treatments for addiction are virtually nonexistent, new strategies to manage these problems are urgently needed (Benowitz, 1993; Marzuk et al., 1995; Carroll et al., 1999). One attractive idea is to speed metabolism of cocaine into nontoxic derivatives by administration of a suitable enzyme (Gorelick, 1997). Human plasma butyrylcholinesterase (BChE) does hydrolyze cocaine into less toxic products, but slowly and inefficiently (Stewart et al., 1977; Inaba et al., 1978). By introducing a single mutation, alanine 328-tyrosine, Xie et al. (1999) succeeded in increasing the rate of cocaine hydrolysis by a factor of 4. Subsequently, in search of a still faster and more potent enzyme, we used molecular modeling of BChE-cocaine complexes to guide further protein engineering (Sun et al., 2001). This approach led to the discovery of two useful mutations, alanine 328-tryptophan and tyrosine 332-alanine. After these mutations, reaction velocity with cocaine rose 40-fold, as measured by k_{cat} , at the cost of a small rise in K_m (Sun et al., 2002a). The resulting cocaine esterase (CocE) was the first BChE mutant to meet suggested criteria for clinical utility against overdose (Landry et al., 1993). We previously

showed that CocE accelerates cocaine clearance in rats and abolishes cocaine-induced hyperactivity in mice (Sun et al., 2002b). The present study was undertaken to determine whether CocE also antagonizes physiological responses associated with cocaine toxicity. We addressed two questions: 1) will CocE pretreatment reduce cocaine-induced hypertension? and 2) will CocE restore normal blood pressure when injected after cocaine?

Materials and Methods

Preparation of Animals. Animal studies, conducted under a protocol approved by the Mayo Institutional Animal Care and Use Committee, employed male Sprague-Dawley rats weighing 250 to 350 g (Harlan, Indianapolis, IN). For continuous monitoring of blood pressure, the rats were anesthetized with sodium pentobarbital (45 mg/kg i.p., Abbott Diagnostics, Abbott Park, IL) or, in another series of experiments, with urethane (1.45 g/kg i.p.). A PE-50 cannula was then placed in the femoral artery on one side and connected to a pressure transducer (Gould TA240; Gould Instrument Systems Inc., Cleveland, OH). A second cannula for drug administration was introduced into the lateral tail vein. Animals remained anesthetized for the duration of the experiment. Core body temperature was maintained within narrow limits by a heating lamp. After surgery, animals were allowed 30 min to stabilize before drug administration.

Cocaine Hydrolase. Recombinant CocE, a doubly mutated version of human BChE (A328W/Y332A rQ45), was prepared in a stable, predominantly tetrameric form. This was accomplished by bulk culture of CHO K1 cells cotransfected with cDNAs for the mutant BChE

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ABBREVIATIONS: BChE, butyrylcholinesterase; CocE, cocaine hydrolase; ANOVA, analysis of variance.

and a portion of the rat COLQ gene (Krejci et al., 1997) as described previously (Altamirano and Lockridge, 1999; Xie et al., 1999). Secreted enzyme was purified by affinity chromatography on procainamide-Sepharose eluted with 0.2 M procainamide, followed by ion exchange chromatography on DE52 eluted with a NaCl gradient in 20 mM Tris-HCl, pH 7.5. All steps during cell culture and protein purification were conducted with scrupulous attention to avoiding contamination with bacterial endotoxin. Purified CocE was dialyzed, concentrated to 9 mg/ml, filter sterilized, and stored at 4°C.

Drug Administration. All drugs were prepared in 0.9% NaCl (saline) and administered through the lateral tail vein. Atropine sulfate (1 mg/kg; Sigma-Aldrich, St. Louis, MO), was injected 10 min before cocaine challenge; (-)-cocaine hydrochloride (typically 7 mg/kg) (Sigma-Aldrich), was given as a rapid infusion over a period of 10 s (total volume, 5 ml/kg). CocE (3 mg/kg) was given either before or after cocaine challenge (total volume, 2 ml/kg). In the control group, an equivalent volume of saline was injected instead of enzyme. Blood pressure was recorded continuously during the injection of drug and for 10 min thereafter.

Tissue Collection and Enzyme Assay. Blood was drawn from the tail vein, collected in heparinized tubes, and centrifuged at once to obtain plasma samples, which were stored at -80°C. The rats were then perfused through the aorta with 250 to 350 ml of saline, which was drained from the left atrium to remove residual blood from lung as well as other tissues. Tissues were frozen at -80°C until assay (completed within 24 h). Thawed tissues were homogenized in 5 volumes of 50 mM sodium phosphate buffer, pH 7.4 with 0.5% Tween 20; supernatants were obtained after centrifugation at 10,000g for 10 min. To evaluate CocE activity in plasma and tissue samples, we used the radiometric assay of Brimijoin et al. (2002) based on release of ³H-benzoic acid from ³H-cocaine followed by selective partitioning from acid medium into toluene-based fluor for liquid scintillation counting. Assays ran at a final cocaine concentration of 18 μM, the K_m value for CocE. A related procedure was used to determine plasma concentrations of ¹⁴C benzoic acid after metabolite injection. Plasma BChE activity was measured by the method of Ellman et al. (1961) with 1 mM butyrylthiocholine as substrate and, as acetylcholinesterase inhibitor, 1, 5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (10⁻⁵ M, Sigma-Aldrich).

Sucrose Density Gradient Fractionation. Molecular forms of CocE were separated on 5-ml gradients of sucrose (5%–20%) in 50 mM Tris-HCl (pH 7.4), 1 M NaCl, and 0.2 mM EDTA. Samples, with catalase as a sedimentation marker, were centrifuged for 16 h at 35,000 rpm (SWi55.1 rotor; Beckman Coulter, Fullerton, CA). Numbered fractions (200 μl) recovered from the bottoms of the tubes were assayed for cocaine hydrolase activity.

Statistical Analysis and Pharmacokinetics. Mean blood pressure during the 10-min period immediately after cocaine or saline injection in control and experimental groups was compared and analyzed statistically with StatView 4.5 (Abacus Concepts, Berkeley, CA). Effects of treatment on the time course of pressor responses were subjected to two-way analysis of variance with time and treatment as factors; a probability of $p < 0.05$ was considered statistically significant.

Enzyme plasma concentration-time profiles were analyzed with a curve-fitting routine in Sigma Plot 4.1 (SPSS Inc., Chicago, IL). Volume of distribution at steady state was calculated using standard noncompartment methodology. Apparent rate constants for redistribution (α) and elimination (β) were calculated along with the associated concentration parameters (A and B) by fitting plasma CocE level to the equation: $CocE = Ae^{-\alpha t} + Be^{-\beta t}$.

Results

Stability and Distribution of CocE. After CocE was injected through the tail vein (3 mg/kg), BChE activity in plasma rose 10-fold; this excess activity then disappeared in

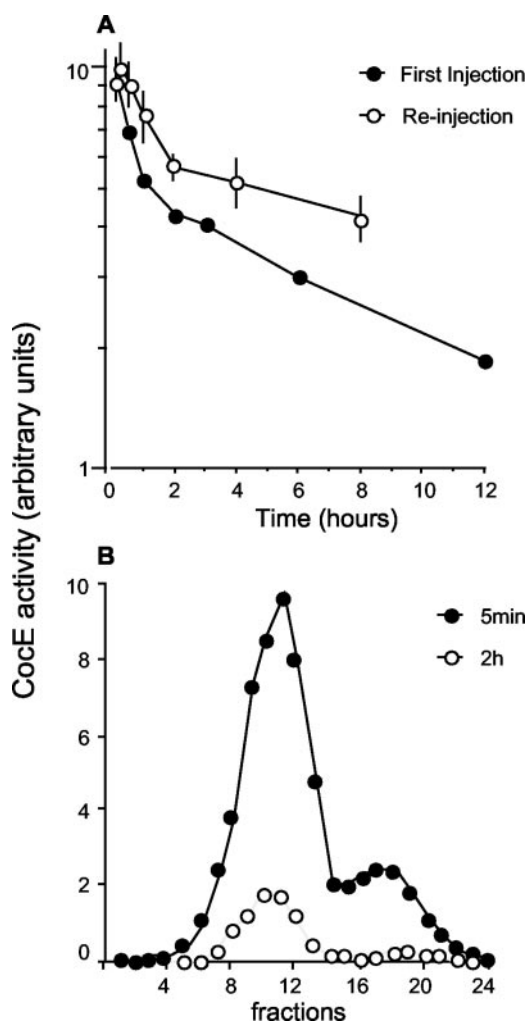


Fig. 1. Stability and “redistribution” of CocE in vivo. A, at time 0, highly purified CocE in a dose of 3 mg/kg was injected into the tail veins of seven rats; after 2 h, plasma was collected and frozen. Later, 2.5 ml of plasma from each “donor rat” was injected into the tail vein of a naive recipient rat. Blood samples (100 μl) were taken from the latter rats at the indicated times and monitored for BChE activity (open circles). Data (means ± S.E.M.) were normalized to match starting activities from rats sampled previously at similar times (filled circles; data from Sun et al., 2002). Actual mean activity in the first sample after reinjection was 0.35 U/ml (about 15-fold lower than the donor plasma). B, sucrose density gradient analysis of CocE molecular forms in plasma from donor rats sampled at indicated times. Activities are means of duplicate samples assayed for cocaine hydrolase activity, expressed in arbitrary units. Top of gradient is toward the right. Fractions 9 to 12 correspond to the G4 or tetrameric form.

a biexponential fashion (Fig. 1A). The estimated half-lives were 22 min for the initial phase and 10 h for the terminal phase, and the calculated volume of distribution was 160 ml/kg. The latter figure was about 3 times greater than the expected plasma volume, although CocE molecules are too large to escape from most capillaries. Binding to red blood cells could not explain the low initial plasma level of CocE because 100% of the activity added to a sample of heparinized whole blood was still present in plasma separated after 1 h of incubation at 37°C. Hence, we tested the possibility that the initial “redistribution phase” might reflect rapid loss of unstable enzyme rather than penetration into tissue. Ultracentrifugation on sucrose density gradients revealed that monomeric CocE (a less stable form) was more abundant in plasma

harvested immediately after enzyme injection than in plasma sampled 2 h later (Fig. 1B). To investigate the issue further, plasma was collected from seven rats when the rapid phase had run its course, 2 h after injection of CocE (3 mg/kg). This plasma was then reinjected into seven naive rats, from which multiple blood samples were obtained at times ranging from 5 min to 8 h. The initial and terminal half-lives for excess BChE activity in those samples were both longer than before (39 min and 28 h), but the estimated volume of distribution was not reduced (163 ml/kg), and the kinetics of enzyme removal remained biphasic (Fig. 1A). Thus, degradation of unstable forms does not explain all of the early drop in plasma CocE content.

A plausible added contributor to rapid "redistribution" would be adsorption to the vascular endothelium. To evaluate this possibility, five rats were given 3 mg/kg enzyme. Two h later, after removal of blood by cardiac perfusion, solid tissues were collected for assay (Fig. 2). Tissue-bound CocE activity was appreciable in heart, lung, kidney, diaphragm muscle, liver, and spleen, but not in brain. Therefore, local adsorption probably did contribute to the rapid loss of injected enzyme from the circulating blood. On the other hand, we noted that no tissue had more than 5% as much activity as an equivalent volume of plasma. Hence, tissue adsorption may not have been the only source of loss.

Prolonging the Transient Pressor Response to Cocaine. In preparation for testing the physiological effects of CocE, baseline cardiovascular responses to cocaine were examined in four naive rats under pentobarbital anesthesia. Three rats showed a brief rise in both systolic and diastolic pressure after receiving 7 mg/kg cocaine, although one did not. Overall, mean blood pressure rose by 25 ± 2.4 mm Hg at

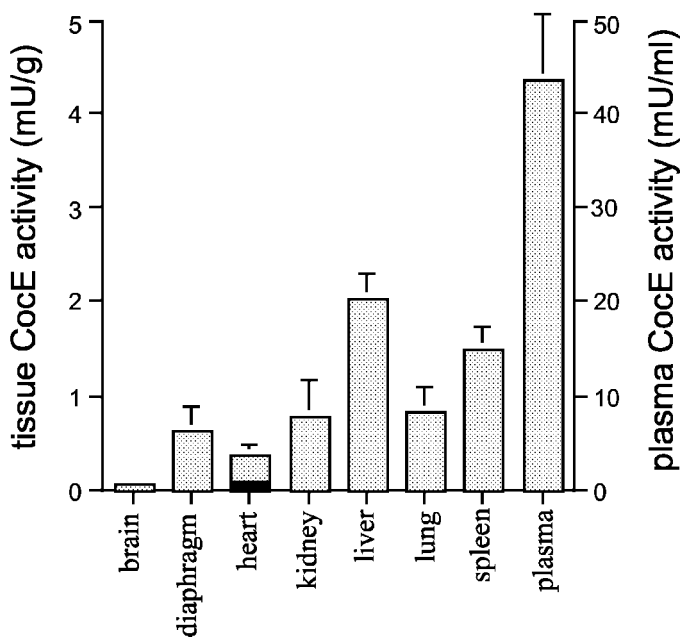


Fig. 2. Tissue retention of injected CocE. Five rats were euthanized 2 h after i.v. injection of 3 mg/kg CocE. To reduce or eliminate enzyme associated with plasma or red blood cells, tissues were collected after 250 to 350 ml of isotonic NaCl (matching the rat's total body mass) was perfused through the aorta. Cocaine hydrolase assays were performed on tissue homogenates and plasma (note different ordinate scales). Means \pm S.E.M. are shown. Black shading indicates cocaine hydrolase activity in untreated control rats (negligible except in heart, which is the richest source of endogenous BChE among the tissues assayed).

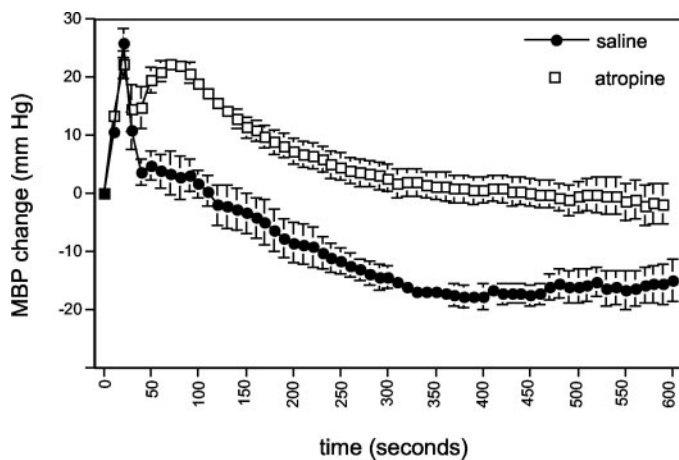


Fig. 3. Prolonging cocaine's pressor response. Pentobarbital anesthetized rats were treated with i.v. atropine 1 mg/kg (open squares, $n = 13$) or saline solution (filled circles, $n = 4$) and 10 min later received i.v. cocaine (7 mg/kg) at zero time. Means \pm S.E.M. of the cocaine-induced changes in mean blood pressure are shown in mm Hg.

20 s after injection, but it returned to baseline at 40 s and slowly declined below normal for several additional minutes (Fig. 3). The duration of this effect appeared less than optimal for studies on reversal of cardiovascular toxicity. The accompanying fall in heart rate was even shorter lived (data not shown). To determine whether longer lasting and more consistent results could be obtained under other conditions, further experiments were carried out on rats anesthetized with urethane. Cocaine raised blood pressure more substantially in these rats (37 ± 3.8 mm Hg), and 90% of them showed increases of at least 15 mm Hg. The pressor response was still brief, however.

With the aim of prolonging pressor responses, rats were pretreated with 1 mg/kg atropine. This step was expected to block muscarinic actions of acetylcholine released from vagal terminals in response to baroreceptor reflexes (Brown and Taylor, 2001). Regardless of the anesthetic, atropine-pretreated rats gave a longer and clearly biphasic pressor response, with a secondary peak between 50 and 100 s after cocaine and a slow return to baseline around 300 s (Fig. 3). The slowing of heart rate, however, still lasted only about 60 s (data not shown). The secondary pressor response varied steeply with cocaine dosage. It was minimal at 1 mg/kg, substantial at 3 mg/kg, and only slightly greater at 7 mg/kg (Fig. 4). Since 7 mg/kg is close to the cocaine LD_{50} in rats, higher doses were not tested. We concluded that pressor responses in atropine-pretreated rats would be a useful paradigm for further studies on the cardiovascular effects of cocaine and their modification by CocE.

Blunting Pressor Responses by CocE Pretreatment. Experiments to test whether enzyme pretreatment would blunt the pressor effects of cocaine were performed on rats anesthetized with urethane to ensure consistency and minimize group sizes. These rats were given 1 mg/kg atropine, followed in 10 min by saline or 3 mg/kg CocE. Four minutes later, 7 mg/kg cocaine was injected. CocE treatment did not affect the brief fall in heart rate after cocaine challenge (Fig. 5), but it strongly affected pressor responses (Fig. 6). Compared with unprotected animals, rats pretreated with CocE showed significantly smaller and shorter increases in mean blood pressure. There was a noticeable drop in the initial

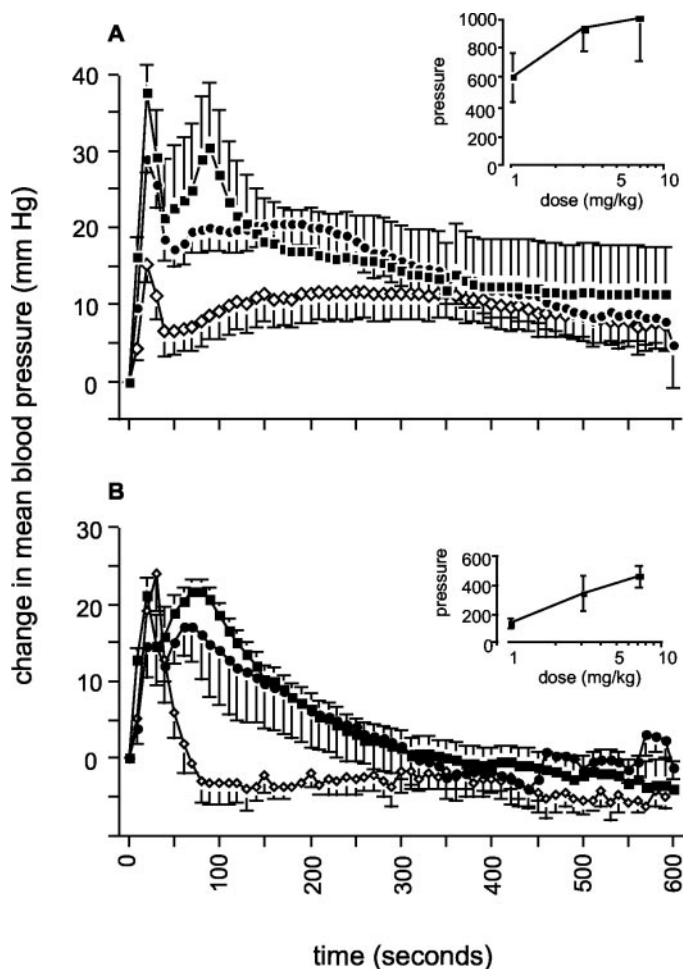


Fig. 4. Dose response relations of pressor effect. Cocaine was administered at zero time to atropine-pretreated rats under anesthesia with urethane (A) or pentobarbital (B), and mean blood pressure was recorded at 10-s intervals. Shown are means \pm S.E.M. of pressor responses to cocaine at 1 mg/kg (open diamonds), 3 mg/kg (filled circles), and 7 mg/kg (filled squares). Group sizes ranged from 5 to 13 rats. Insets show the relation between dose and total area under the pressor-response curves.

pressure peak at 20 s and an even larger decrease in the secondary peak. On the other hand, CocE alone, in rats that received no cocaine, caused no measurable change in mean blood pressure or heart rate. Thus, CocE specifically blunted pressor responses rather than being hypotensive itself.

Antipressor effects of CocE in these experiments might have reflected some action on blood vessels or baroreceptors instead of, or in addition to, accelerated breakdown of cocaine. To evaluate this possibility, we tested CocE with the natural sympathetic neurotransmitter, norepinephrine, a directly acting pressor agent. Under conditions identical to those of the previous experiment, however, pretreatment with CocE caused little change in pressor response (Fig. 7). Thus, the antipressor effects of CocE appear to be selective and consistent with an accelerated metabolic transformation of cocaine.

Reversing Established Pressor Responses by "CocE Rescue." It remained to be seen whether enhanced cocaine metabolism could easily reverse an established cardiovascular action. In an attempt to demonstrate this point, we delayed CocE treatment until pressor responses to cocaine had fully developed. The injections were timed to coincide with

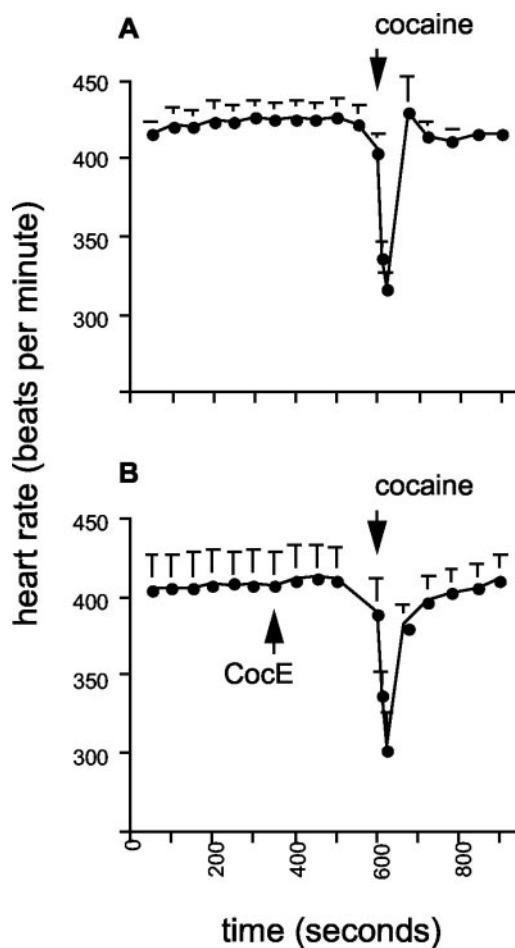


Fig. 5. Cocaine effects on heart rate. Heart rate was monitored at 50 s intervals in atropine-treated rats under urethane anesthesia. CocE (3 mg/kg) and cocaine (7 mg/kg) were injected at the indicated times (arrows). Means \pm S.E.M. are shown (group sizes eight to nine). In other rats under pentobarbital anesthesia, cocaine induced about one-third less bradycardia than illustrated here. A, cocaine injection without enzyme pretreatment. B, cocaine injection after enzyme pretreatment. The degree of bradycardia in the two treatment groups did not differ significantly.

the peak of blood pressure, identified as the first point at which pressure remained constant for a 10-s interval. For these experiments, we reverted to pentobarbital anesthesia (as preferred by our Department of Veterinary Medicine). Cocaine (7 mg/kg) was administered 10 min after atropine. Because pressor responses under pentobarbital were not uniform, we studied only rats showing a peak increase of 15 mm Hg or greater in mean blood pressure. Roughly one-third of tested animals failed on this criterion and were euthanized immediately. In the remaining subjects, CocE (3 mg/kg) or a comparable volume of saline was injected at the pressure peak, and cardiovascular parameters were monitored for the next 10 min. Alignment of the data using enzyme injection as the zero time point revealed a dramatic and highly significant reversal of the pressor response to cocaine (Fig. 8). Hypertension in saline-injected rats was sustained for 5 min or more, but pressure in CocE-treated rats normalized within 60 s and remained at or below baseline during the entire window of observation.

On average, peak pressor responses followed cocaine injection with the same delay in the 13 enzyme-treated and the 11 control rats (113 ± 19 and 109 ± 29 s, respectively). How-

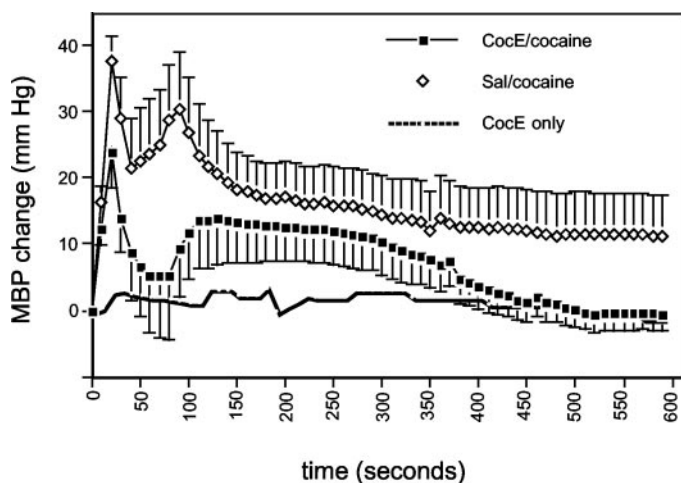


Fig. 6. CocE pretreatment reduces cocaine response. Atropine-treated rats under urethane anesthesia were given CocE (3 mg/kg i.v.) or saline solution, 4 min before injection of cocaine (7 mg/kg) at zero time. Means \pm S.E.M. of changes in blood pressure are shown at 10-s intervals after drug challenge (group sizes eight to nine). The overall effect of CocE treatment was highly significant by two-way ANOVA ($p < 0.0001$), and the initial pressure peak was significantly reduced ($p < 0.05$).

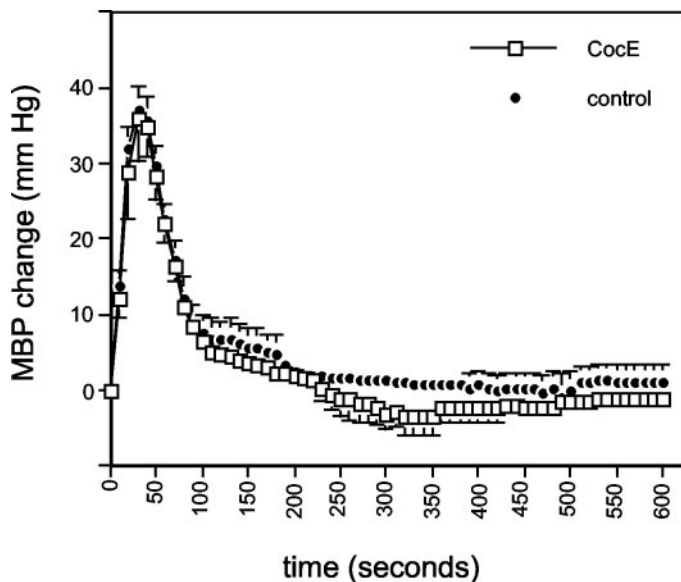


Fig. 7. CocE pretreatment does not alter response to norepinephrine. Atropine-treated rats under urethane anesthesia (four per group) were given CocE (3 mg/kg i.v.) or saline solution, 4 min before injection of norepinephrine (6 μ g/kg) at zero time. Means \pm S.E.M. of changes in blood pressure are shown at 10-s intervals. No treatment effect was detected by two-way ANOVA during the initial 300 s.

ever, because the outcome might still have been affected by variations in the timing of CocE injection, a separate analysis was performed on a balanced subset of seven animals per group, treated with a smaller range of delays (70–150 s, mean = 108 ± 7). The data from these 14 rats again indicated dramatic pressor reversal ($p < 0.01$ by two-way ANOVA). These results leave little doubt that CocE not only blunts responses to subsequent cocaine exposure but can quickly reverse established responses as well.

The speed of pressor reversal and the eventual development of a slight hypotension raised the possibility that products of cocaine hydrolysis might have helped lower blood pressure. For that reason, we tested the pressor effects of an

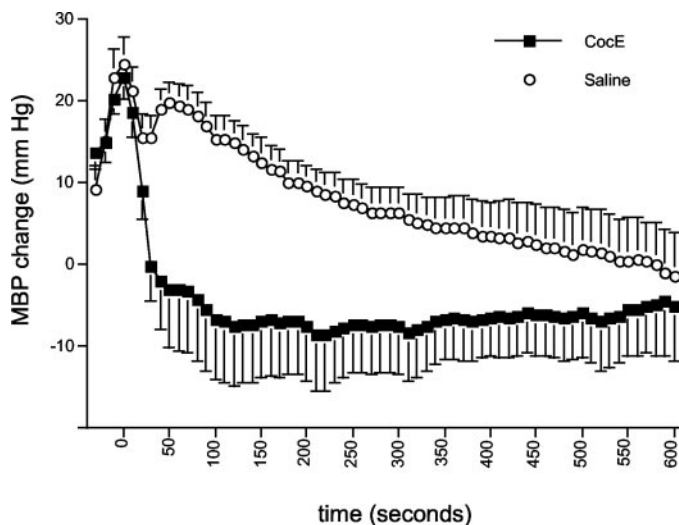


Fig. 8. CocE reversal of established pressor effect. Atropine-treated rats under pentobarbital anesthesia were challenged with cocaine (7 mg/kg). When blood pressure was maximal, CocE (3 mg/kg i.v.) or saline solution was administered (group sizes 13 and 11, respectively). Shown here are means \pm S.E.M. of changes in pressure from the moment of cocaine injection. Data from all 24 rats were aligned with CocE injection as zero time (see text). The effect of CocE treatment was significant by two-way ANOVA ($p < 0.0001$).

equimolar mixture of the two metabolites, ecgonine methyl ester and benzoic acid, each given at 5 μ mol/kg. This dose was selected because, in plasma drawn 2 min after injection, it raised the benzoic acid level to 5 μ M, the concentration previously found 2 min after CocE-treated rats received 7 mg/kg cocaine (Sun et al., 2002b). However, when blood pressure was monitored in two atropine-pretreated rats, the mixture of cocaine metabolites caused no detectable hypotension unless the dose was doubled. Furthermore, when three other rats received cocaine together with 5 μ mol/kg metabolites, the normal pressor response was not reduced (data not shown). Thus, a sudden burst of circulating metabolites does not appear to explain CocE's rapid reversal of hypertension after cocaine challenge.

Discussion

Reversibility of Cocaine's Cardiovascular Effects in Vivo. We have found that an efficient cocaine hydrolase will reduce hypertensive responses to cocaine in the rat and that such an enzyme can be beneficial even after responses to the drug have become established. It is particularly important that an injection of CocE restored baseline blood pressure in rats that received a near-LD₅₀ dose of cocaine. This finding is directly relevant to the clinical utility of hydrolases for rescue from cocaine overdose.

The rapid restoration of normotension shows that cocaine's binding to its sites of action is reversible in vivo on a time scale of seconds. Our data confirm previous indications that cocaine's high affinity for its target, the catecholamine transporter, is compatible with ready reversibility. Such reversibility is expected. For example, even the potent muscarinic antagonist, *N*-methylscopolamine, with K_d of 0.2 nM, dissociates from its preferred receptor subtype with a rate constant of 0.027 min^{-1} and a half-life of 25 s (McKinney, 1998). Cocaine's affinity for the norepinephrine transporter is lower and its dissociation half-life should be correspondingly

shorter. The behavior of this drug, however, is complicated by a tendency to sequester within cells and in body lipids. One might suppose that cocaine flowing down concentration gradients from these storage sites into plasma would partly offset metabolic losses in the bloodstream. To some extent, cocaine must shift from tissue depots to plasma. This effect probably explains why a dose of CocE that clears cocaine from isolated plasma in 2 min requires over 15 min for clearance in vivo (Sun et al., 2002b). Nonetheless, our data establish the key point that modest amounts of CocE will substantially shorten cocaine's plasma half-life, blunt pressor responses to a delayed injection of cocaine, and reverse a fully developed hypertension.

Why Was Hypertension So Rapidly Reversed? One surprise in these experiments was that CocE restored normal blood pressure within 3 min, whereas in our earlier study on similarly treated rats, plasma cocaine levels remained above 1 μM for at least 5 min (Sun et al., 2002b). In testing CocE and cocaine metabolites for hypotensive properties that might explain such results, we saw no effects at realistic doses. It is worth noting that the dose-response relationship of cocaine and blood pressure was steep, especially in rats under pentobarbital anesthesia. We cannot exclude the possibility that the reduction of blood pressure might be disproportionate to the reduction in drug level or that the intensity of response might be affected by the rate of metabolic change. Another relevant factor could be the deposition of active CocE in solid tissues, as demonstrated in our experiment on enzyme distribution. This localized hydrolase could impede the access of cocaine to its cardiovascular targets. Adsorbed enzyme might have been even greater than measured in our tissue samples, since loosely bound CocE could have dissociated during the perfusion intended to eliminate blood-borne material.

Finally, CocE must be considered as a site for instantaneous adsorption of circulating cocaine. Although plasma cocaine levels never reached the CocE K_m (18 μM), enzyme concentration in the injected material was about 1 μM . Therefore, the initial bolus might have bound minor amounts of drug.

Why Did CocE Not Prevent Changes in Heart Rate? Cocaine injection was typically accompanied by a transient fall in heart rate, lasting 30 to 40 s. The primary mechanism of this effect is likely to be the activation of baroreceptors in response to cocaine-induced hypertension. As a result, impulse traffic to the cardiac pacemaker should rise in sympathetic pathways while falling in vagal pathways. In rats treated with atropine to block vagal function, the reduction of sympathetic traffic should be most important. It is not obvious to us why a CocE pretreatment that reduced cocaine-induced hypertension did not also prevent cocaine-induced bradycardia. Possible explanations, which require additional experiments for their evaluation, include local anesthetic and other direct effects of cocaine or its metabolites on the pacemaker.

Using Cocaine Hydrolases to Treat Drug Toxicity. Sufficient doses of unmodified human BChE have been found to protect rodents against cocaine without exerting harmful effects of their own. Thus, native human enzyme in amounts up to 28 mg/kg prevented or reduced cocaine toxicity in rats and mice as measured in terms of hypertension, cardiac arrhythmia, locomotor activity, and death by seizure (Hoff-

man et al., 1996; Lynch et al., 1997; Mattes et al., 1997; Carmona et al., 1998). Prior studies have also shown that BChE from horse serum will accelerate cocaine metabolism even in primates (Carmona et al., 2000). In general, however, beneficial effects have required 400- to 800-fold increases of plasma BChE activity. With CocE treatment, equivalent or larger increases in the rate of cocaine hydrolysis occur at far lower doses of enzyme. Not only does this potency offer practical advantages for clinical application, it also facilitates experimental studies of "metabolic therapies" for cocaine overdose in animal models.

Further experiments are needed to define the true potential for reversal of cocaine toxicity by rescue therapy with a cocaine hydrolase and to determine the relative importance of tissue-bound enzyme. Additional preclinical pharmacology must simulate cocaine intoxication as realistically as possible and should also consider possible differences arising from administration by inhalation as opposed to injection. It is well recognized that anesthetics exert differential influences on the sympathetic nervous system (Ebert, 1990; Charney et al., 2001), which in turn is crucial in determining cocaine's cardiovascular effects (Trendelenburg, 1966; Jain et al., 1990). The anesthetic chosen for most of our experiments was urethane, under which the cardiovascular effects of cocaine resemble those in conscious animals (Pitts et al., 1987). However, there is no question that the presence of anesthetics is a complicating factor and that pentobarbital in particular can change the intensity and direction of cocaine responses. Therefore, we need data from unanesthetized animals. Future studies should also identify long-lived indices of cocaine toxicity for paradigms that model the clinical crisis presented by a patient arriving in the emergency room 30 to 60 min after overdose. Such indices may include EKG and electroencephalogram abnormalities since arrhythmia and seizure are characteristic features of cocaine intoxication (Benchimol et al., 1978; Przywara and Dambach, 1989; Pascual-Leone et al., 1990; Beckman et al., 1991; Winbery et al., 1998).

Newer Hydrolases for Use in Patients. The greatest promise for rapid drug clearance after overdose lies with "third generation" hydrolases engineered for the greatest possible catalytic efficiency with cocaine. Our original molecular modeling of interactions between BChE and cocaine stereoisomers (Sun et al., 2001) provided a basis for rational mutation of this enzyme to improve catalysis. Landry and colleagues have just confirmed those results in an essentially duplicate study (Zhan et al., 2003). More significant is the recent discovery, by a team in the biotechnology industry, of a superior BChE derivative that incorporates one of the mutations in CocE, plus several others (Pancook et al., 2003). This enzyme, designated AME-359, has an enhanced k_{cat} for cocaine and is roughly 6-fold more efficient than CocE or about 250-fold more efficient than native human BChE. We predict that AME-359 in a dose of 0.5 mg/kg will offer cardiovascular protection equaling or surpassing that seen in the present experiments with CocE at 3 mg/kg. To put this into perspective, total therapeutic doses for an 80-kg individual might be 10,000 mg of native BChE, 240 mg of CocE, or 40 mg of AME-359. In our view, the first is impractical, the second is possible, and the third is reasonable.

Compared with enzymes from nonhuman species, derivatives of human BChE have advantages of relatively low immunogenicity in patients. No antibody response was detected

after intraspecific injection of purified macaque BChE (Rosenberg et al., 2002). A reduced chance of immune responses is one reason to prefer CocE or AME-359 for clinical use over a highly active bacterial cocaine hydrolase recently reported by Bresler et al. (2000). Native human BChE is now being considered for therapeutic applications like protecting military personnel against organophosphorus anticholinesterases and related "nerve agents" (Doctor, 2003). Efforts to validate this concept have confirmed the safety of BChE. That is hardly surprising since BChE as a natural constituent of human plasma is administered to patients with every pint of whole blood and, to our knowledge, has never caused a transfusion reaction. In fact, partially purified human BChE has been administered to patients on a daily basis over periods of up to several weeks without ill effect (Stovner and Stadskleiv, 1976; Cascio et al., 1982; Ostergaard et al., 1988). Because the mutated residues in CocE and AME-359 lie primarily inside the catalytic gorge, they are unlikely to confer significant immunogenicity. Of course, additional investigation is warranted. For the present, however, there is reason for optimism that administration of modified human BChE will become, at the least, a useful added weapon in combination therapy for cocaine overdose. Such treatment might be especially advantageous for individuals with atypical forms of BChE, a phenotype that confers an elevated K_m for cocaine hydrolysis (Xie et al., 1999) and, most likely, an elevated risk of toxicity.

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