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Cocaine Esterase: Interactions with Cocaine and Immune Responses in Mice

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Abstract

Cocaine esterase (CocE) is the most efficient protein catalyst for the hydrolysis of cocaine characterized to date. The aim of this study was to investigate the *in vivo* potency of CocE in blocking cocaine-induced toxicity in the mouse and to assess CocE's potential immunogenicity. Cocaine toxicity was quantified by measuring the occurrence of convulsions and lethality. Intravenous administration of CocE (0.1-1 mg) 1 min prior to cocaine administration produced dose-dependent rightward shifts of the dose-response curve for cocaine toxicity. More important, i.v. CocE (0.1-1 mg), given 1 min after the occurrence of cocaine-induced convulsions, shortened the recovery time following the convulsions, and saved the mice from subsequent death. Effects of repeated exposures to CocE were evaluated by measuring anti-CocE antibody titers and the protective effects of i.v. CocE 0.32 mg against toxicity elicited by i.p. cocaine 320 mg/kg (i.e., 0-17% occurrence of convulsions and lethality). CocE retained its potency against cocaine toxicity in mice following a single prior CocE exposure (0.1-1 mg), and these mice did not show an immune response. CocE retained similar effectiveness in mice following three prior CocE exposures (0.1-1 mg/week x 3), although these mice displayed 10-fold higher antibody titers. CocE partially lost effectiveness (i.e., 33-50% occurrence of convulsions and lethality) in mice with four prior exposures to CocE (0.1-1 mg/2 weeks x 4) and these mice displayed approximately 100-fold higher antibody titers. These results suggest that CocE produces robust protection and reversal of cocaine toxicity, indicating CocE's therapeutic potential for acute cocaine toxicity. Repeated CocE exposures may increase its immunogenicity and partially reduce its protective ability.

Introduction

Cocaine abuse continues to be a serious public health problem in the United States (NIDA, 2004). A recent National Survey on Drug Use and Health indicates that an estimated 1.6 million Americans could be classified as dependent on or abusing cocaine in the past 12 months in 2004 (SAMHSA, 2005). The same survey estimates that there were 2 million current (past-month) users of cocaine (SAMHSA, 2005). More important, data from the Drug Abuse Warning Network showed that cocaine-related emergency department visits increased 126% between 1995 and 2004, rising from 58 to 131 visits per 100,000 people. Cocaine was first on the list of illicit-drug-related emergency department visits (SAMHSA, 2006).

Sequelae of cocaine overdose include generalized clonic-tonic seizures and status epilepticus capable of producing long-term neurological impairment and death (Kramer et al., 1990; Benowitz, 1993). Cocaine-induced seizures can be resistant to anticonvulsants such as benzodiazepines and are considered to be a major determinant of cocaine-related lethality (Dhuna et al., 1991; Benowitz, 1993). Unfortunately, there is no effective treatment for cocaine abuse and toxicity, and the search for effective and safe treatments continues (Dickerson and Janda, 2005; Sofuoglu and Kosten, 2005; Vocci et al., 2005).

Cocaine blocks the reuptake of dopamine, norepinephrine, and serotonin following binding to monoamine transporters (Hoffman et al., 1991; Kilty et al., 1991; Pacholczyk et al., 1991; Ramamoorthy and Blakely, 1999). In addition, cocaine produces local anesthetic effects through its blockade of sodium channels (Nettleton and Wang, 1990; Wright et al., 1997; O'Leary and Chahine, 2002). Cocaine's primary

action for causing cardiac arrhythmias and sudden death may be its blockade of sodium and potassium channels (Crumb and Clarkson, 1990; Zhang et al., 2001; Bauman and DiDomenico, 2002; Wilson and Shelat, 2003). The inherent difficulties in selectively targeting different receptor/ion channel sites that correspond to the multiple sites of actions of cocaine have led to the development of protein-based therapeutics. One approach to reducing the effects of cocaine is to eliminate it quickly by administration of esterases that rapidly metabolize cocaine. Butyrylcholinesterase (BChE), the major cocaine-metabolizing enzyme present in the plasma of humans and other mammals (Lynch et al., 1997; Mattes et al., 1997), and a bacterial cocaine esterase (CocE) (Bresler et al., 2000; Cooper et al., 2006) have been explored as potential enzymatic therapeutics.

CocE was originally identified in the bacterium *Rhodococcus* sp. strain MB1 which grows in the rhizosphere soil of the cocaine-producing plant *Erythroxylum coca* (Bresler et al., 2000). The bacterium uses cocaine as its sole source of carbon and nitrogen by synthesizing CocE to initiate metabolism of cocaine. CocE is a globular, 574-amino acid enzyme with a molecular weight of ~65 kDa and is the most efficient protein catalyst for the hydrolysis of cocaine characterized to date (Bresler et al., 2000; Larsen et al., 2002; Turner et al., 2002; Rogers et al., 2005). The hydrolytic rate constant of this enzyme (k_{cat}/K_m) is 1000-fold higher than that of BChE, and 10^5-fold and 10^6-fold faster than catalytic antibodies such as Mab 15A10 (Deng et al., 2002; Turner et al., 2002). A recent *in vivo* study in rats has further demonstrated CocE's superior catalytic efficiency and selectivity for cocaine compared with BChE (Cooper et al., 2006). In particular, i.v. CocE 1 mg with 1-min pretreatment protected 100% of rats

receiving i.p. cocaine 180 mg/kg, but i.v. BChE 13 mg (i.e., a 10-fold multiple of the molar equivalent dose of CocE) failed to protect rats from cocaine-induced lethality (Cooper et al., 2006).

Although the catalytic efficiency of CocE makes it an ideal candidate for an improved therapy for cocaine acute toxicity, it has been speculated that this bacterial enzyme would be rapidly cleared via proteolysis and immune surveillance (Rogers et al., 2005). CocE was found to have a remarkably short half-life (i.e., approximate 10 min) in the rat plasma and accordingly to have a short duration of protective effects (Cooper et al., 2006). Given CocE's rapid clearance and poor thermostability, it is possible that CocE may have reduced immunogenicity. It is important to investigate to what degree CocE exposure is liable to produce an immune response and how CocE's immunogenicity affects CocE's ability to protect against cocaine toxicity. The aim of this study was therefore to investigate whether CocE could prevent and reverse cocaine-induced toxicity in mice and whether repeated exposures of CocE could increase immunologic responses and change the effectiveness of CocE *in vivo*.

Materials and Methods

Subjects

Male NIH-Swiss mice (25-32 g) were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and were housed in groups of 6 mice per cage. All mice were allowed *ad libitum* access to food and water, and were maintained on a 12-h light-dark cycle with lights on at 06.30 AM in a room kept at a temperature of 21-22 ^{o}C. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocols were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Procedures

I. Behavioral Assays.

Cocaine-induced toxicity was characterized by the occurrence of convulsions and lethality. Cocaine-induced convulsions were defined as loss of righting posture for at least 5 s with the simultaneous presence of clonic limb movements (Gasior et al., 2000; Daniels et al., 2006). Lethality was defined as cessation of observed movement and respiration. Following i.p. cocaine administration, mice were immediately placed individually in Plexiglas containers (16x28x20 cm high) for observation. The presence or absence of convulsions and lethality and the time to affected responses were recorded for 60 min following cocaine administration.

II. Intravenous Administration.

The mouse was placed in a small restraint chamber (Outer tube diameter: 30 mm, Inner tube diameter: 24 mm, Model #BS4-34-0012, Harvard Apparatus, Inc., Holliston, MA) that left the tail exposed. The tail was cleansed with an alcohol wipe and a 30G1/2 precision glide needle (Fisher Scientific, Pittsburgh, PA) was inserted into one of the side veins for infusion. The i.v. injection volume of CocE was 0.2 mL per mouse. To staunch the bleeding, sterile gauze and pressure were applied to the injection site.

III. Serum Collection.

Mice have a large vein draining the eye and submandibular area which meet at the rear of the cheek pouch. This vein provides a convenient and consistent source of blood (i.e., cheek-pouch blood sampling). A mouse bleeding lancet, (GoldenRod 4.0 mm animal lancet, MEDIpoint Inc., Mineola, NY), was used to puncture this submandibular vein. The blood was collected in a tube from the puncture and was prepared as serum samples (50 μL/mouse) for CocE antibody titer determinations. As soon as the blood was collected, sterile gauze and pressure were applied at the puncture site to minimize the bleeding, and the mouse was returned to its home cage.

IV. Immunological Determination.

A direct ELISA specific for anti-CocE antibodies was set up using a standard protocol. CocE was used (1 μg/mL) to coat a 96-well micro-titer plate using borate buffered saline (1.5 M NaCl, 0.5 M H3BO3, 1.0 M NaOH) to resuspend CocE (50 μL/well). The coating plates were left overnight at 4 ^{o}C. The coating buffer was removed the following morning and the plates blocked with 2% normal goat serum in phosphate-buffered saline (PBS) for 1 h at 37 ^{o}C and washed 3 times. Serum from the various groups of mice was serially diluted in 50 μL of PBS in the wells in a range of 10^2 to 10^7

and run in duplicate. The plates were covered and incubated for 1 h at 37 ^{o}C. Subsequently, the plates were washed 3 times and 50 μL/well of goat anti-mouse IgG peroxidase labeled antibody diluted 1:400. The plates were then washed 3 times and 100 μL peroxidase substrate solution (OPD dissolved in citrate/phosphate buffer) was added to each well. After a 5-10 min incubation (based upon color development in the positive controls), the reaction was stopped using 3M H2SO4 (50 μL/well). The plates were read at 490 nm and titer was determined by the highest dilution that showed increases over background absorbance. Positive controls were derived by immunizing Balb/c mice with 100 μg in 100 μL of CocE emulsified in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, St. Louis, MO) by i.p. administration.

Experimental Designs.

The first part of the study was to determine the ability of CocE to protect and reverse cocaine-induced toxicity. In the protection study, CocE (0.1, 0.32, and 1 mg) was administered i.v. 1 min before administration of several doses of i.p. cocaine (180, 320, 560, 1000, 1800 mg/kg). Dose-response curves of cocaine-induced convulsions and lethality in the absence or presence of different doses of CocE were determined to demonstrate the *in vivo* protective effects of CocE. After establishing a dose-response of CocE, the dosing condition of i.p. cocaine 320 mg/kg in the presence of i.v. CocE 0.32 mg was chosen to study the time course of CocE's protective effects. Prevention of cocaine toxicity by CocE was determined by using different CocE pretreatment time points (i.e., 1, 5, 10, and 20 min before cocaine administration). In the rescue study, CocE (0.1, 0.32, and 1 mg) was administered i.v. within the first min after the

occurrence of convulsions induced by i.p. cocaine 100 mg/kg. This cocaine dose was chosen because it produced convulsions in 100% of mice and lethality in 40-60% of mice based on our pilot study. The interval between the onset of convulsion and lethality was approximately 4-5 min which allowed an opportunity for CocE administration to rescue the mouse from cocaine (i.e., 100 mg/kg) intoxication.

The second part of the study was to determine how prior exposure to CocE evoked immunological responses and how immunogenicity affected CocE's protection against cocaine toxicity. Three CocE dosing regimens were used to investigate whether CocE retained its effectiveness following repeated administration and whether anti-CocE antibody titers increased accordingly. The experimental condition of i.p. cocaine 320 mg/kg in the presence of i.v. CocE 0.32 mg (1-min pretreatment) was chosen to determine CocE's ability to prevent cocaine toxicity. Anti-CocE antibodies were determined from serum samples collected 24 h before each toxicity test. The first dosing regimen gave mice a single i.v. exposure to CocE (0, 0.1, 0.32, or 1 mg) and CocE's effectiveness was assessed in these pretreated mice one month later. The second dosing regimen gave mice three i.v. exposures to CocE (0-1 mg/week x 3; once per week for three weeks) and determined CocE's effectiveness one week later. One additional group was used to immunize mice by using 0.1 mg of CocE in conjunction with complete and incomplete Freund's adjuvants (CFA and IFA) (Week 1: i.p. CocE 0.1 mg + s.c. CocE 0.1 mg + i.p. CFA 0.1 mg + s.c. CFA; Week 2: i.p. CocE 0.1 mg + i.p. IFA 0.1 mg; Week 3: i.p. CocE 0.1 mg + i.p. IFA 0.1 mg; Week 4: serum collection and behavioral toxicity test) in order to investigate whether a large increase in the titer number significantly reduced CocE's effectiveness in immunized mice. The third dosing

regimen gave mice four i.v. exposures of CocE (0-1 mg/2 weeks x 4; once per two weeks for 8 weeks) and determined CocE's effectiveness in these pretreated mice two weeks later.

Data analysis.

Data from the behavioral toxicity studies (i.e., % of mice showing affected responses) were analyzed with Fisher's exact probability test with one tail. The values of $ED_{100conv}$ and LD_{100} were determined when 100% of mice (n=6-8) showed convulsions or death, respectively. These values were used to compare the degree of rightward shifts of cocaine's dose-response curve in the absence or presence of CocE pretreatment. In addition, mean values (mean ± S.E.M.) were calculated from individual values for other endpoints (i.e., time to affected responses and the titer numbers). These data were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple (*post hoc*) comparisons. The criterion for significance was set at $p<0.05$.

Drugs.

Cocaine hydrochloride (Mallinckrodt Inc., St. Louis, MO) was dissolved in sterile water and was administered intraperitoneally at a volume of 0.01 mL/g. CocE (purified and supplied by Drs. D. Narasimhan and R.K. Sunahara, see details in Cooper et al., 2006) was diluted to difference concentrations in PBS and administered intravenously at a volume of 0.2 mL/mouse.

Results

Intraperitoneal administration of cocaine dose-dependently produced convulsions ($ED_{100conv}$: 100 mg/kg) and lethality (LD_{100}: 130 mg/kg) in mice (Figure 1, left panels). The intervals between cocaine administration and onset of convulsions or death were 2.4 ± 0.3 min (mean ± S.E.M.) and 5.4 ± 0.5 min, respectively, after cocaine 130 mg/kg, yielding a convulsion-death interval of about 3 min (Figure 1, right panels). The intervals between cocaine administration and onset of convulsions or death were 1.5 ± 0.2 min and 3.0 ± 0.2 min, respectively, after cocaine 180 mg/kg, yielding a convulsion-death interval of about 1.5 min.

Pretreatment with CocE (i.e., 1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced convulsions and lethality (Figure 1, left panels). In particular, CocE 0.32 mg and 1 mg produced 10- and 18-fold shifts, respectively, in the dose-response curve for cocaine-induced convulsions, such that 1000 and 1800 mg/kg cocaine (i.e., doses of $ED_{100conv}$) were required to surmount the protective properties of CocE. Likewise, CocE 0.32 mg and 1 mg produced approximately 8- and 14-fold shifts in the dose-response curve for cocaine-induced lethality by increasing the LD_{100} dose to 1000 and 1800 mg/kg, respectively. Although 0.32 mg and 1 mg of CocE did not protect mice receiving 1000 or 1800 mg/kg of cocaine, the time to the measured responses after cocaine administration was increased significantly ($p<0.05$, Figure 1, right panels).

Pretreatment with CocE produced a time-dependent protection against cocaine-induced convulsions and lethality (Figure 2). One hundred percent and 67% of mice were saved when i.v. CocE 0.32 mg was administered 1 and 5 min, respectively, before

administration of i.p. cocaine 320 mg/kg. CocE's protective effects were completely reduced when mice received CocE 20 min before cocaine administration.

The ability of CocE to reverse cocaine-induced toxicity was studied after mice had received i.p. cocaine 100 mg/kg and displayed convulsions. All four groups of mice showed convulsions and the intervals between cocaine administration and onset of convulsions among groups were similar (Figures 3A & 3B). Intravenous administration of CocE not only shortened the time to the recovery from convulsions (Figure 3C), it also saved all mice from subsequent death (Figure 3D; i.e., compared with the PBS-treated group showing 50% occurrence of lethality). The intervals between cocaine administration and onset of convulsions or death were 2.9 ± 0.3 min and 6.8 ± 1.2 min, respectively, after cocaine 100 mg/kg, yielding a convulsion-death interval of about 4-5 min in the PBS-treated group (Figures 3B and 3E).

Effects of repeated exposures to CocE were evaluated by measuring the protective effects of i.v. CocE 0.32 mg against toxicity elicited by i.p. cocaine 320 mg/kg and the antibody titers (Figures 4-6). One prior CocE exposure (0.1-1 mg) did not change the ability of CocE to prevent cocaine toxicity as i.v. CocE 0.32 mg with 1-min pretreatment saved 83-100% of mice receiving i.p. cocaine 320 mg/kg (Figure 4, top two panels). In addition, this dosing regimen did not increase anti-CocE antibody titers (Figure 4, bottom panel). Three prior CocE exposures also did not reduce the effectiveness of CocE as CocE retained its ability to protect cocaine toxicity. I.v. CocE 0.32 mg saved 100% of mice receiving i.p. cocaine 320 mg/kg (Figure 5, top two panels). Nevertheless, this dosing regimen produced a 10-fold increase in the anti-CocE antibody titers irrespective of the dose of CocE given (Figure 5, bottom panel). In

contrast, mice immunized with Freund's adjuvant showed a 1000-fold increase of antibody titers and CocE lost its ability to prevent cocaine-induced convulsions and lethality (i.e., the rightmost bars in Figure 5).

Four prior CocE exposures reduced the effectiveness of CocE as CocE partially lost its ability to protect cocaine toxicity. I.v. CocE 0.32 mg saved 50-67% of mice receiving i.p. cocaine 320 mg/kg (Figure 6, top two panels). In addition, this dosing regimen produced 100- to 1000-fold increases in the anti-CocE antibody titers irrespective of CocE dose (Figure 6, bottom panel). Figure 7 provides a depiction of the relation between antibody titer and responses of each mouse receiving prior exposures to CocE. The top panel shows the convulsive responses of each mouse to i.p. cocaine 320 mg/kg in the presence of i.v. CocE 0.32 (1 min pretreatment) and each mouse's antibody titer value determined by ELISA (in log unit) after different dosing regimens of CocE. The bottom panel shows the survival or death of each mouse and the real value of the antibody titer after different dosing regimens of CocE. Both panels illustrate each mouse's measured responses and its antibody titer following different CocE prior exposure conditions shown in Figures 4, 5, and 6.

Discussion

This study showed that i.v. pretreatment with CocE dose-dependently protected mice from cocaine-induced convulsions and lethality. A dramatic shift (i.e, 10-20 fold) to the right in the dose-response curve of cocaine in producing convulsions and lethality was afforded by both 0.32 and 1 mg of CocE. These dose-dependent rightward shifts produced by CocE are profound. No other reported agents are able to shift the dose-response curve for cocaine toxicity to this extent (Gasior et al., 2000; Carrera et al., 2005; Daniels et al., 2006). These findings further support the first *in vivo* study of CocE in rats demonstrating CocE's rapid and robust protection against cocaine-induced lethality (Cooper et al., 2006). It will be valuable to investigate whether other effects of cocaine such as cardiovascular and local anesthetic effects can be antagonized by CocE. Interestingly, i.v. BChE 13 mg (i.e., a 10-fold multiple of the molar equivalent dose to CocE) failed to protect rats from i.p. 180 mg/kg of cocaine-induced lethality (Cooper et al., 2006). In our pilot study, BChE 1 mg also failed to protect against the lethality produced by 320 mg/kg of cocaine in this mouse toxicity assay (unpublished data). BChE only protected rats from cardiovascular changes and convulsions elicited by small to moderate doses of cocaine (1 to 80 mg/kg: Lynch et al., 1997; Mattes et al., 1997). Several BChE mutants with improved catalytic efficiency have been engineered (Pan et al., 2005). It will be important to study and compare protective effects of these BChE mutants with those of CocE in the rodent model of cocaine overdose.

The duration of CocE's protection against cocaine toxicity is short. CocE given 10 min before cocaine only protected 50% of mice from cocaine lethality (Figure 2). Although the duration of CocE's esteratic activity in the mouse plasma is unknown,

CocE was found to have a short half-life (~10 min) in the rat plasma (Cooper et al., 2006). Preliminary *in vitro* data also suggested that CocE undergoes a temperature-dependent inactivation with a $t_{1/2}$ of approximately 15 min at 37 ^{o}C (Mierzejewski and Sunahara, personal communication). These findings may in part explain the fact that CocE not only decreased the potency of cocaine in producing convulsions and lethality, but also prolonged the time to exhibit convulsions and lethality when 100% of mice with CocE pretreatment were affected (Figure 1). Nevertheless, CocE's short duration of action should not detract from its potential in the treatment of acute cocaine overdose, although it probably prevents its usefulness in the treatment of cocaine abuse (i.e., self-administration). A compound that has any use in the treatment of drug abuse, particularly if it acts to eliminate the action of the abused drug, must have an extended duration of action. It is possible and important to design a CocE mutant with improved thermostability by using combinational chemistry and amino acid mutation.

A pivotal finding from this study is that CocE given *after* the occurrence of convulsions not only shortened the duration of convulsions, but also saved mice from subsequent lethality (Figure 3). Previous studies have shown that post-cocaine administration of cocaine-metabolizing enzymes such as, BChE and CocE, can protect against cocaine toxicity (Lynch et al., 1997; Mattes et al., 1997; Cooper et al., 2006). What is novel in the present study is that CocE can reverse cocaine toxicity when it is given at a time point *after* the occurrence of convulsions (i.e., post-cocaine vs. post-cocaine-convulsions). As mentioned previously in the Methods, i.p. cocaine 100 mg/kg provided only a 4-5 min window of opportunity for CocE's rescue. The temporal profile of the mouse's responses to cocaine overdose does not simulate the situation of

emergency department visit which will have a longer interval between cocaine overdose and treatment. It would be valuable to assess CocE's ability to reverse the effects of cocaine in non-human primates before initiating clinical trials.

Although both *in vitro* and *in vivo* studies have demonstrated that CocE is the most efficient protein catalyst for hydrolyzing cocaine characterized to date (Larsen et al., 2002; Turner et al., 2002; Cooper et al., 2006), CocE is a large, bacterial protein, and as such can be expected to produce an immune response (Rogers et al., 2005). Surprisingly, a single prior exposure of CocE did not elicit a significant antibody response and did not change CocE's effectiveness (Figure 4). Three prior CocE exposures once per week slightly increased the anti-CocE antibody titers, but CocE retained its ability to protect these mice from cocaine toxicity (Figure 5). Immunization using CFA followed by IFA has been shown to enhance antibody responses (Alving et al., 1995; Koetzner et al., 2001; Shu et al., 2001). As a positive control group, mice immunized with Freund's adjuvant showed a large increase in the antibody titers (i.e., 1000-fold) and CocE completely lost its ability to protect these immunized mice (i.e., 100% of mice died following cocaine administration). These findings may indicate that CocE is a weak antigen and can maintain its protective ability following multiple exposures.

However, four prior CocE exposures once per two weeks significantly increased the antibody titers and CocE partially lost its ability to protect mice from cocaine toxicity (i.e., 33-50% of mice exhibited convulsions and died) (Figure 6). This regimen produced 100- to 1000-fold increases in the anti-CocE antibody titers, which were close to those observed in mice immunized with Freund's adjuvant. Interestingly however, not every

mouse expressing a 1000-fold higher titer exhibited reduced protective effects of CocE (Figure 7). It is possible that CFA/IFA immunization promotes development of more efficient antibodies. Nevertheless, these experiments indicate that reduction of CocE's protective ability is correlated to the antibody titer elicited with multiple doses of CocE. Future studies using repeated administration of cocaine combined with post-injection of CocE can further address the question of whether other factors such as damage to the myocardium following each cocaine overdose exposure can affect the usefulness of repeated CocE in the treatment of cocaine toxicity.

In summary, this study demonstrates that CocE dose-dependently protects and reverses cocaine-induced convulsions and lethality in mice and provides *in vivo* evidence for the therapeutic potential of CocE in the treatment of acute cocaine toxicity. Nevertheless, repeated CocE exposures increased the risk of immunological effects and, in part, reduced CocE's protective ability. This functional study provides a pharmacological basis for future research and development of CocE mutants or/and pegylated CocE mutants that may have greatly improved thermostability and reduced immunogenicity (Harris and Chess, 2003). More important, the study provides the first evidence of using CocE as a treatment modality in acute cocaine toxicity after the induction of convulsions.

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Footnotes

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Legends for Figures.

Figure 1. Protective effects of CocE against cocaine-induced toxicity. CocE (mg) was administered intravenously 1 min before cocaine administration (mg/kg, i.p.). Left panels: Dose-response curves of cocaine-induced convulsions and lethality in the absence or presence of CocE. Each data point represents the percentage of mice (n=6 for each dosing condition) exhibiting cocaine-induced convulsions or lethality. Right panels: Time to toxic effects of cocaine in the absence or presence of CocE in the dosing condition that 100% mice showed affected responses. Each value represents mean ± S.E.M. (n=6). The asterisks represent a significant difference from the condition of cocaine alone (∗, $p<0.05$). See *Materials and Methods* for other details.

Figure 2. Time course of protective effects of CocE against cocaine toxicity. Each data point represents the percentage of mice (n=6 for each dosing condition) exhibiting cocaine-induced convulsions or lethality. CocE (0.32 mg, i.v.) was administered 1, 5, 10, and 20 min before cocaine administration (320 mg/kg, i.p.). The asterisks represent a significant difference from the 20-min CocE pretreatment group showing 100% affected (∗, $p<0.05$). See Figure 1 for other details.

Figure 3. Effects of CocE in reversing cocaine-induced toxicity. CocE (mg) was administered intravenously within the first min after the occurrence of convulsions induced by i.p. cocaine 100 mg/kg. Panels A and B: The occurrence of convulsions and the interval between cocaine administration and onset of convulsions after mice receiving cocaine 100 mg/kg. Panel C: Time to the recovery from convulsions after

CocE administration. Panels D and E: The occurrence of lethality and the interval between cocaine administration and onset of death in mice with or without post-injection of CocE. Each data point represents the percentage of mice (n=8 for each dosing condition) exhibiting cocaine-induced convulsions or lethality. Each value represents mean ± S.E.M.. The asterisks represent a significant difference from the group not receiving CocE (i.e., PBS-treated group, ∗∗, $p<0.01$; ∗, $p<0.05$). See *Materials and Methods* for other details.

Figure 4. Effects of a single prior CocE exposure on the effectiveness of CocE. Protective effects of i.v. CocE (0.32 mg, 1 min pretreatment) against i.p. cocaine 320 mg/kg were tested in mice 1 month after they received a single i.v. administration of CocE. The anti-CocE antibody titers were determined from serum samples collected 24 h before the toxicity test. Each value represents the percentage of mice affected or the mean ± S.E.M. (n=6).

Figure 5. Effects of three prior CocE exposures on the effectiveness of CocE. Protective effects of i.v. CocE (0.32 mg, 1 min pretreatment) against i.p. cocaine 320 mg/kg were tested in mice 1 week after they received 3 exposures of i.v. CocE of various doses (0-1 mg/week x 3). The rightmost bars (i.e., 0.1/FA) illustrate effects of CocE 0.1 mg in conjunction with CFA/IFA under the same dosing regimen. The antibody titers were determined from serum samples collected 24 h before the toxicity test. Each value represents the percentage of mice affected or the mean ± S.E.M.

(n=6). The asterisks represent a significant difference from the group not receiving prior CocE exposures (∗∗, $p<0.01$; ∗, $p<0.05$). See *Materials and Methods* for other details.

Figure 6. Effects of four prior CocE exposures on the effectiveness of CocE. Protective effects of i.v. CocE (0.32 mg, 1 min pretreatment) against i.p. cocaine 320 mg/kg were tested in mice 2 weeks after they received repeated i.v. CocE (0-1 mg/2 weeks x 4). The antibody titers were determined from serum samples collected 24 h before the toxicity test. Each value represents the percentage of mice affected or the mean ± S.E.M. (n=6). The asterisks represent a significant difference from the group not receiving prior CocE exposures (∗∗, $p<0.01$). See *Materials and Methods* for other details.

Figure 7. Relationship between the antibody titer numbers and affected responses of mice receiving prior CocE exposures. Symbols represent subjects tested in protective effects of CocE against cocaine-induced toxicity under different dosing regimens shown in Figures 4, 5, and 6. Open symbols indicate measured antibody titer number of each mouse that did not exhibit convulsions or lethality. Filled symbols indicate measured antibody titer number of each mouse that exhibited convulsions or/and lethality. See Figures 4, 5, and 6 for other details.

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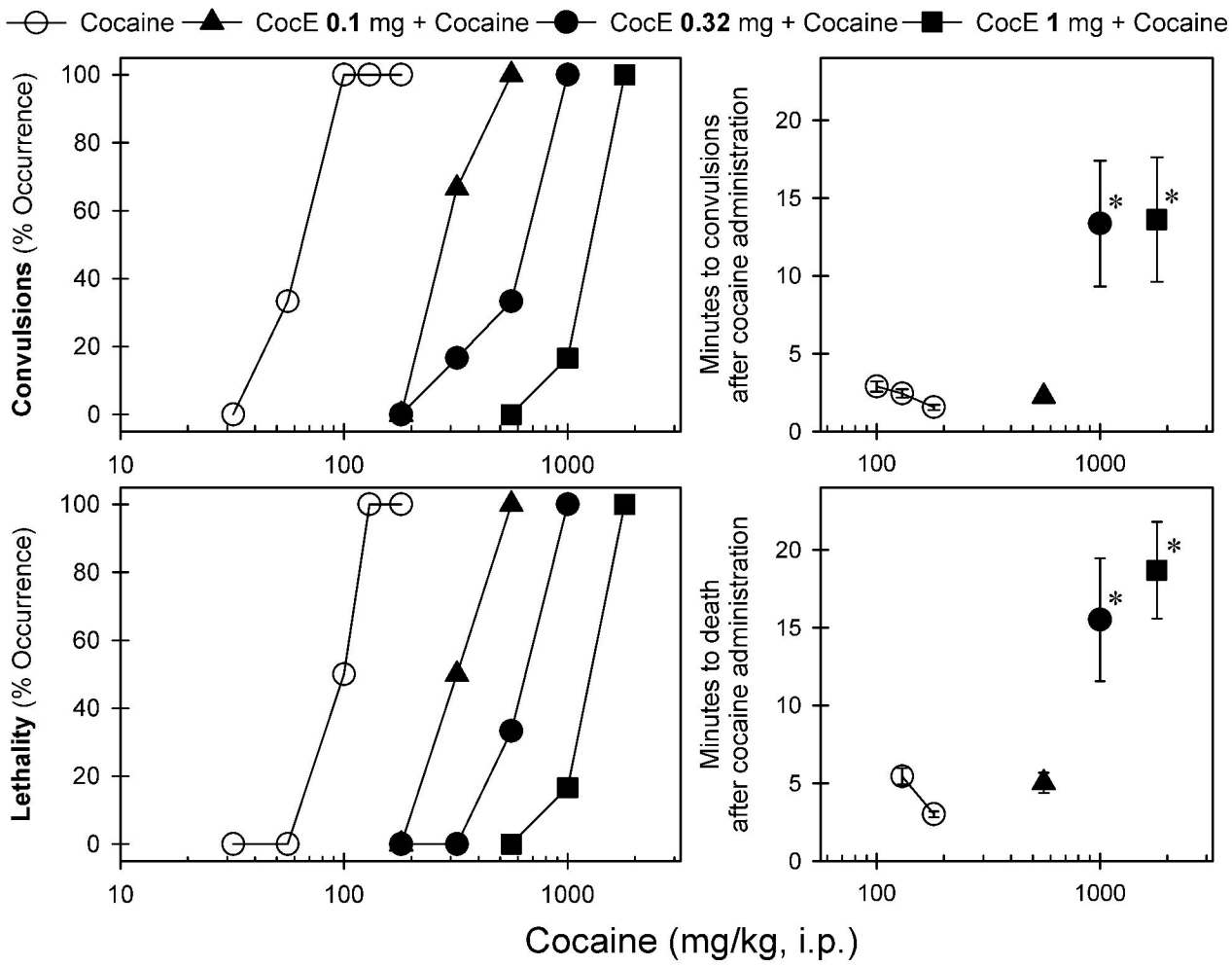

Figure 1

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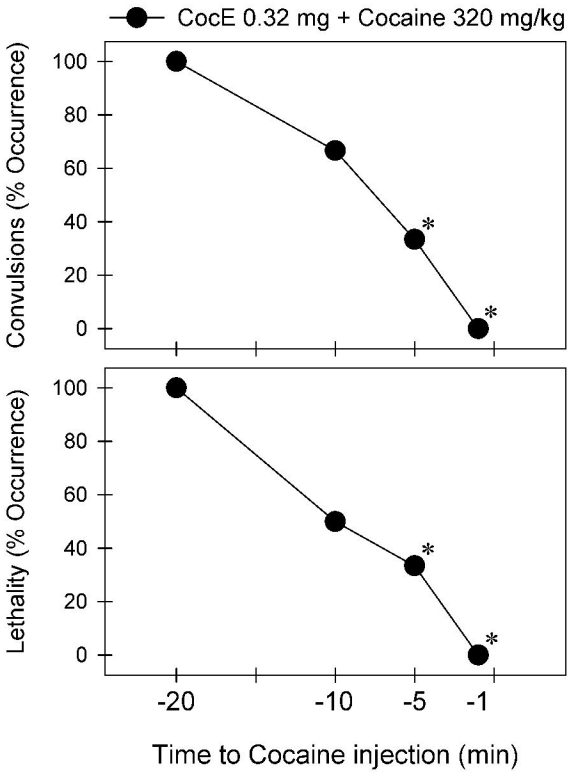

Figure 2

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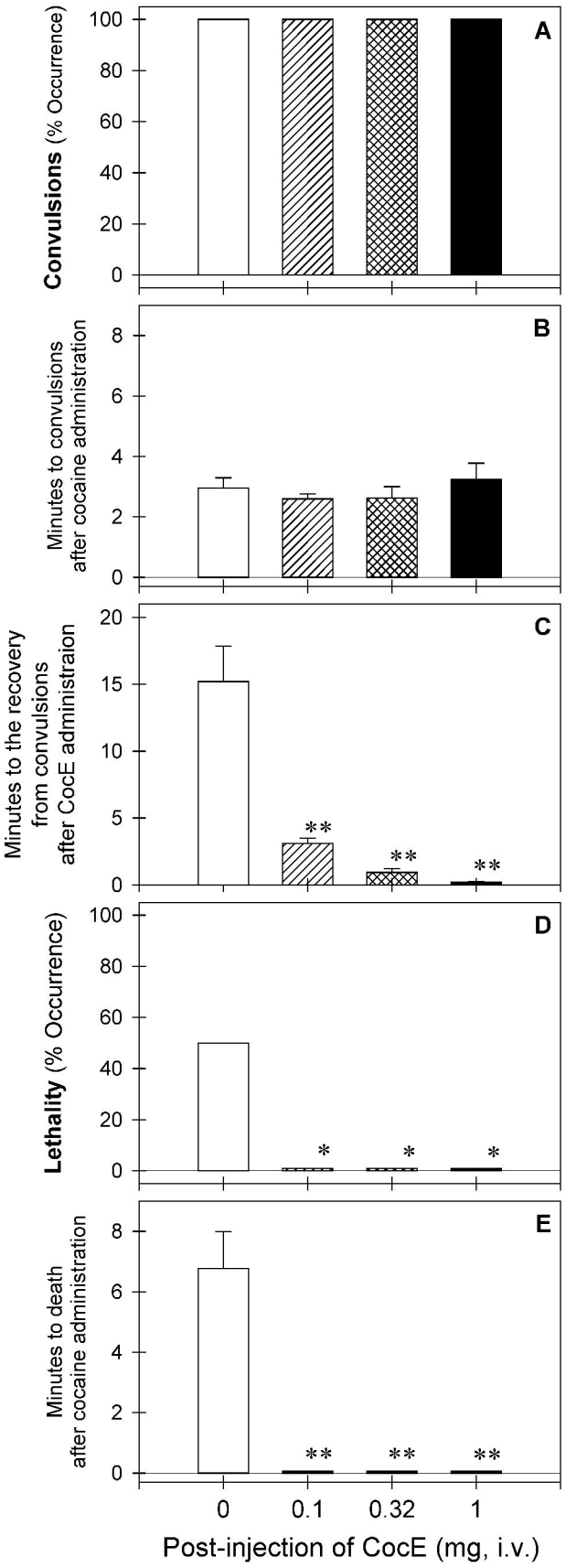

Figure 3

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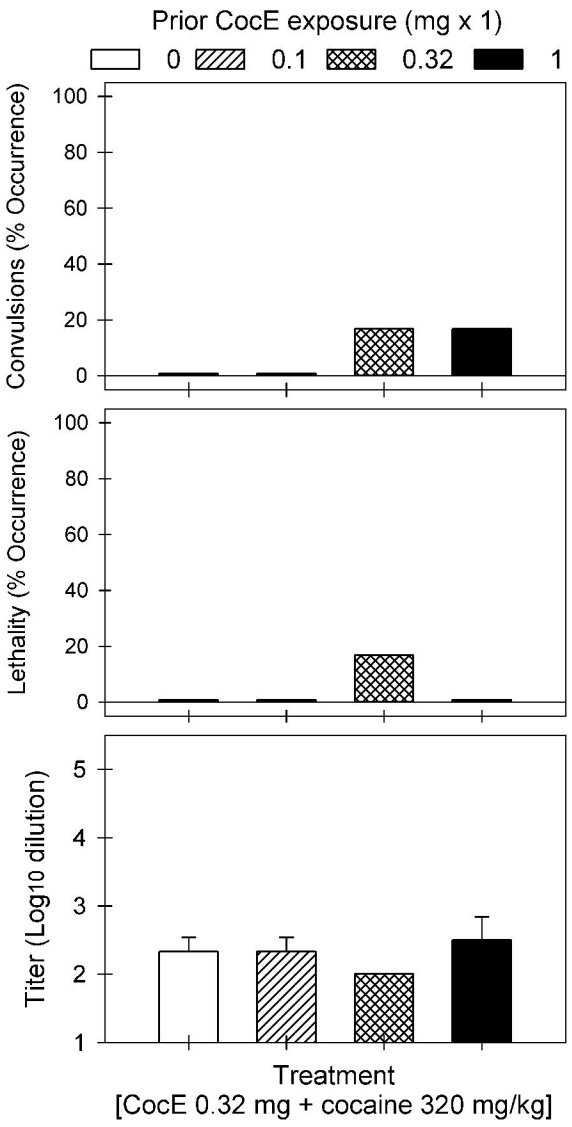

Figure 4

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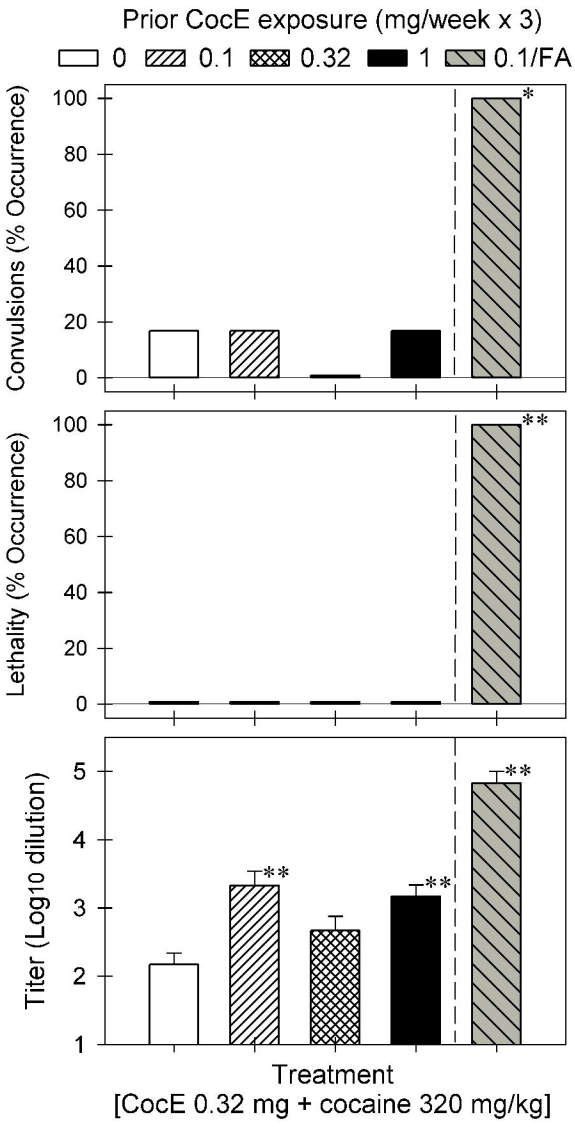

Figure 5

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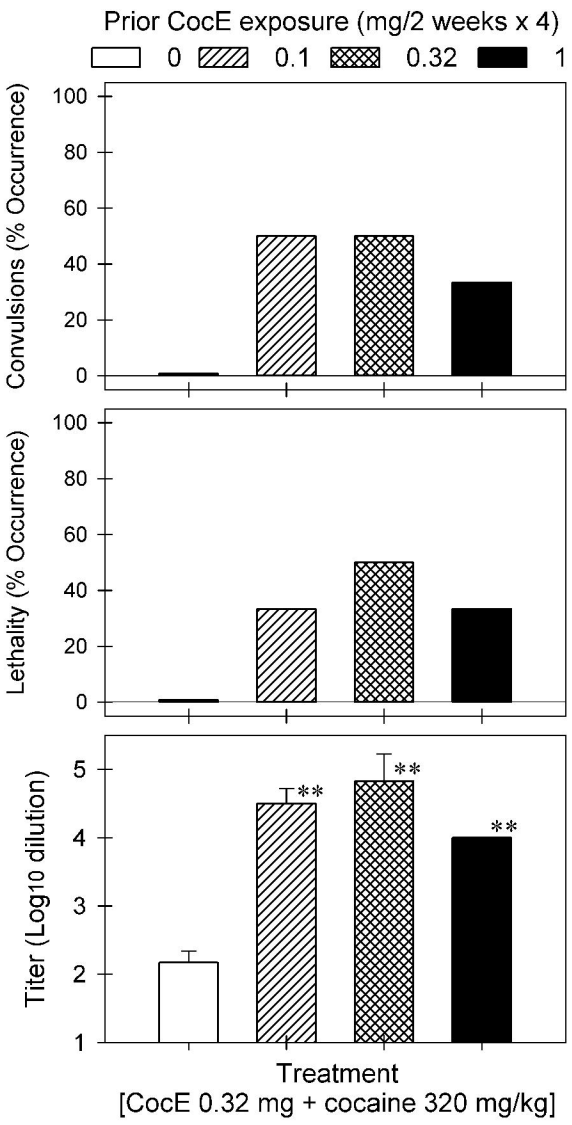

Figure 6

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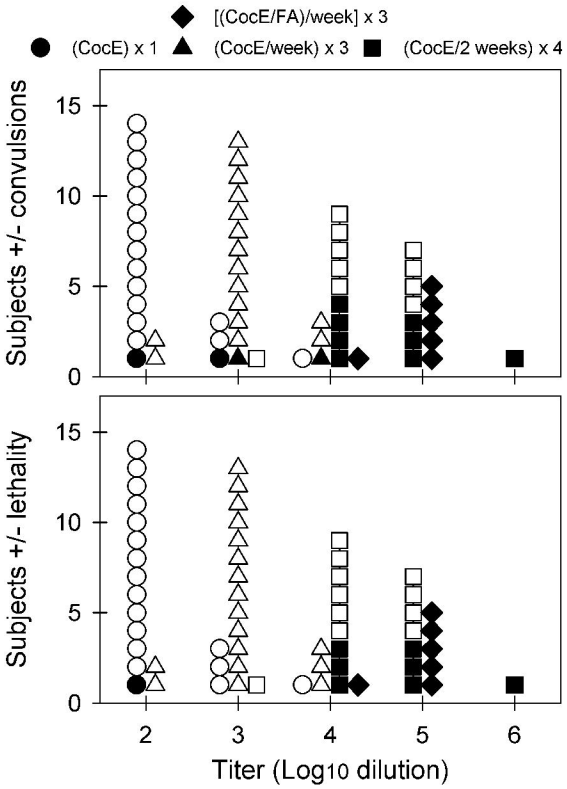

Figure 7