Cocaine Metabolism Accelerated by a Re-Engineered Human Butyrylcholinesterase

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ABSTRACT

Plasma butyrylcholinesterase (BChE) is important in the metabolism of cocaine, but natural human BChE has limited therapeutic potential for detoxication because of low catalytic efficiency with cocaine. Here we report pharmacokinetics of cocaine in rats treated with A328W/Y332A BChE, an excellent cocaine hydrolase designed with the aid of molecular modeling. Compared with wild-type BChE, this enzyme hydrolyzes cocaine with 40-fold improved $k_{cat}$ (154 min$^{-1}$ versus 4.1 min$^{-1}$) and only slightly increased $K_M$ (18 μM versus 4.5 μM). In rats given this hydrolase (3 mg/kg i.v.) 10 min before cocaine challenge (6.8 mg/kg i.v.), cocaine half-life was reduced from 52 min to 18 min. Mirroring the reductions of plasma cocaine were large increases in benzoic acid, a product of BChE-mediated cocaine hydrolysis. All other pharmacokinetic parameters confirmed a large, dose-dependent acceleration of cocaine removal by the injected cocaine hydrolase. These results show that A328W/Y332A, an efficient cocaine hydrolase in vivo as well as in vitro, might promote cocaine detoxication in a clinical setting.

Because classic pharmacological treatments for cocaine overdose are not fully effective (Hollander, 1995), there is reason to consider alternative therapeutic strategies. Although fatal cocaine toxicity can present with widely varying drug levels (Wetli and Wright, 1979), some studies have found a correlation between cocaine response and plasma cocaine concentration (Javaid et al., 1978; Cone et al., 1988; Lau et al., 1991). Also, it has been reported (Hoffman et al., 1992) that cocaine toxicity tends to vary inversely with the levels of plasma butyrylcholinesterase (BChE), a major factor in cocaine metabolism. Such observations led to the idea that enhancing the metabolic conversion of cocaine to less toxic derivatives could be therapeutically useful (Gorelick, 1997). Plasma BChE can hydrolyze cocaine to ecgonine methyl ester and benzoic acid (Stewart et al., 1977) (Fig. 1), which lack the pharmacological activity of cocaine (Madden and Powers, 1990). In doses that increase plasma BChE levels 400-fold, BChE is claimed to enhance cocaine metabolism in monkeys (Carmona et al., 2000). Large quantities of exogenous BChE also protect rodents and monkeys against cocaine toxicity (Hoffman et al., 1996; Lynch et al., 1997; Mattes et al., 1997; Carmona et al., 1998), and even lethal overdose (Hoffman et al., 1996).

In search of a cocaine hydrolase that would have a useful impact at more practical dose levels, novel mutants of human plasma BChE have been explored (Xie et al., 1999). Recently, we performed molecular modeling studies of enzyme-cocaine complexes (Sun et al., 2001) and used the results to engineer a powerful cocaine hydrolase by site-directed mutagenesis of BChE (Sun et al., 2002). The new enzyme, A328W/Y332A, showed 40-fold improvement in $k_{cat}$ over wild-type BChE and only slightly increased $K_M$ (18 μM versus 4.5 μM). This hydrolase is the first BChE mutant whose kinetic properties meet previously suggested criteria for clinical utility in treating cocaine overdose (Landry et al., 1993). Compared with a recently reported bacterial cocaine hydrolase of even higher catalytic efficiency (Larsen et al., 2002), the modified BChE is attractive in that, as a nearly natural human protein, it is less likely to provoke immunological reactions.

We previously observed that A328W/Y332A BChE dramatically accelerates cocaine clearance in isolated plasma and, when injected into mice, it abolishes cocaine-induced hyperactivity (Sun et al., 2002). Here we report effects of A328W/Y332A BChE on plasma cocaine in rats. Although physiological studies have yet to be performed, the pharmacokinetic and metabolic data suggest that treatment with A328W/Y332A (or other cocaine hydrolases with equal or better
ing saline solution were administered to replace lost plasma volume estimated plasma volume in total. Equal volumes of heparin-contain-
(Borchard et al., 1990).

Drug Enforcement Administration, while 3H-(\text{H}11002)-cocaine was purchased from Sigma-Aldrich.

phate, and tetraisopropyl pyrophosphoramide (iso-OMPA), all from Sigma-Aldrich. Other reagents were echothiophate iodide from Wyeth-Ayerst (Rous-
es Point, NY), and butyrylthiocholine iodide, diisopropyl fluorophos-
3M echothiophate, 10

Cocaine Levels in Plasma and Tissue. Animal studies, con-
ducted under a protocol approved by the Mayo Institutional Animal Care and Use Committee, employed male Sprague-Dawley rats weighing 250 to 350 g (Harlan, Madison WI). Under urethane anesthesia (1.45 mg/kg i.p.), catheters were placed in the tail vein (to deliver drugs or BChE) and carotid artery (to sample blood). Rats remained anesthetized for the duration of the experiment and were finally euthanized with sodium pentobarbital (250 mg/kg i.v.). BChE (1 or 3 mg/kg) was administered first. Cocaine injections consisted of unlabeled drug mixed with exactly 30 \mu Ci of \text{[3H]cocaine} (50 Ci/mmol). The dose (6.8 mg/kg i.v. or 60 mg/kg i.p.) corresponded to 40 or 80% of reported LD_{50} values for respective routes of administration (Borchard et al., 1990).

Multiple blood samples were taken, generally less than 10% of estimated plasma volume in total. Equal volumes of heparin-containing saline solution were administered to keep catheters patent. Each sample was collected into an iced centrifuge tube containing inhibitors of BChE and carboxylesterase (10^{-5} M echothiophate, 10^{-5} M iso-OMPA, 25 \mu M saturated sodium fluoride). These conditions were found to prevent measurable hydrolysis of cocaine during the brief interval between sampling and analysis. Inhibitors were omitted when BChE activity was to be determined. Plasma isolated by centrifugation (1500, 10 min) was assayed immediately by a radiometric method for cocaine and benzoic acid or stored at -80°C for mass spectrometry.

Radiometric Assay. To measure cocaine and benzoic acid, the product of cocaine hydrolysis by BChE, we used sensitive radiometric assays based on toluene extraction of \text{[3H]cocaine} labeled on its benzene ring (Sun et al., 2001, 2002). In brief, 200-\mu l plasma aliquots were acidified with 300 \mu l of 0.02 M HCl for extraction of benzoic acid while paired aliquots were alkalinized with 300 \mu l of 1 M Na_{2}CO_{3} for extraction of cocaine. These samples were vigorously mixed for 10 s with 4 ml of toluene-based scintillation fluor. After phase separation by centrifugation, organic phases were collected for scintillation counting. Under the extraction conditions of the cocaine assay, authentic \text{[3H]cocaine} was quantitatively detected, whereas \text{[3H]benzoic acid} was almost undetected (<1%). The reverse was true of the benzoic acid assay. After the results were confirmed by mass spectrometry (see below), the assays were deemed suitable for rapid determination of plasma drug levels.

Liquid Chromatography-Mass Spectrometry (LC-MS). Plasma samples were prepared for LC-MS as described by Singh et al. (1999). Frozen plasma was quickly thawed, mixed, and microcentrifuged for 2 min at 14,000 rpm; 300-\mu l supernatant aliquots were then transferred to clean tubes with 1 ml of acetonitrile, mixed for 10 s, and centrifuged for 10 min at 2,500g, 4°C. Supernatants were transferred into clean tubes with 15 \mu l of formic acid. After vigorous mixing for 10 s, samples were evaporated to dryness at room temperature in a vacuum centrifuge. Dried samples were reconstituted with 1 ml of water and passed through a 0.2-\mu m syringe filter before analysis by LC-MS. Analysis after the fact indicated no appreciable loss of cocaine or metabolites attributable to "online decomposition" of samples waiting in queue.

LC separation was performed on an HP 1100 separator (Agilent Technologies, Palo Alto, CA) equipped with auto-sampler, vacuum degasser, and column heater. Analyte (30 \mu l) was injected onto a 5-mm, 2.0 × 150 mm C18 column (Vydac, Hesperia, CA) with mobile
phases: A, 100:0.1 water/formic acid; B, 100:0.1 acetonitrile/formic acid. A gradient of 0 to 2% phase B over 0.5 min followed by 2 to 90% phase B over 10 min was applied at a rate of 300 µL/min. The entire effluent was introduced into the electrospray interface of an Esquire3000 ion trap mass spectrometer (Bruker Daltonics, Inc. Billerica, MA) and ionized using a capillary voltage of 3500 kV, with an N2 nebulizer gas set at 35 psi and dry gas at 10 l/min. Data were acquired in normal scanning mode over a mass range of m/z 50 to 600 with values of skimmer 1 at 21.3 V, octopole at 2.69 V, and trap drive at 50. Cocaine, norcocaine, benzoylecgonine (BE), and ecgonine methyl ester (EME) were analyzed separately. These compounds showed m/z of 304.0, 290.0, 290.0, and 200.0, respectively, in MS full scan mode. The detection limit is ≤1 ppb/µL for all three compounds. MS/MS revealed major losses of benzoic acid (cocaine, norcocaine, and BE) or water (norcocaine and EME). Norcocaine and benzoylecgonine were distinguished only by the products at m/z 136.0 and 150.0, respectively. Ecgonine itself was not examined but would not have contributed to the measured peaks of parent compound or other metabolites in the MS analysis.

**Statistical Analysis and Pharmacokinetics.** Treatment effects were subjected to analysis of variance using StatView 4.5 (Abacus Concepts, Berkeley, CA). Post hoc testing was based on Fisher’s protected least significant difference; p < 0.05 was considered statistically significant. Cocaine plasma concentration-time profiles were analyzed with WinNonlin (SCI Software, Lexington, KY). Cocaine levels after i.p. administration were described by a one-compartment model because absorption was slower than redistribution. Derived parameters included absorption half-life (0.693/kₐ), peak cocaine concentration (Cmax) and time to peak concentration (tmax). Data from i.v. bolus administration were analyzed by an open two-compartment model, with elimination from the central compartment. Apparent rate constants for redistribution (α) and elimination (β) were calculated along with the associated concentration parameters (A and B) by fitting plasma cocaine level, Cp, to the following equation.

\[ \text{Cp} = \text{A}e^{-\alpha t} + \text{Be}^{-\beta t} \]

Clearance (CL) and volume of distribution at steady state (Vss) were calculated using standard noncompartment methodology. Area under the curve (AUC₁₋₅) and area under the first moment curve (AUMC₁₋₅) from time 0 to final sample were determined by the trapezoidal method. Terminal areas from the last measured concentration (Ct) to infinity were calculated as Ct/β for AUC₁₋₅ and as Ct × α + Ct/β² for AUMC₁₋₅. Total clearance from plasma (CL₁₋₅) was defined as dose/AUC₁₋₅, and V₅ was defined as dose × AUMC₁₋₅/AUC₁₋₅.

**Results**

**Fate of A328W/Y332A in Vivo.** First we determined the distribution and stability of recombinant A328W/Y332A BChE after an i.v. injection (Fig. 2). Two minutes after the injection (0.5 mg/kg), plasma BChE activity with butyrylthiocholine as substrate had increased 80-fold over basal levels. Over the next 12 h, BChE activity decayed with a biphasic exponential course involving a rapid phase (half-life, 22 min) and a slower phase (half-life, ~9 h). These characteristics indicated that BChE activity remained reasonably stable during the first hours after the injection. Interestingly, the apparent steady-state volume of distribution was 48 ml, about 3 times the expected plasma volume of a 300-g rat.

**Pharmacokinetics of Intravenous Cocaine.** Plasma was repeatedly sampled between 2 min and 2 h after a cocaine injection of 6.8 mg/kg, and cocaine levels were monitored by a radiometric assay. From the initial value of 5.8 µM, the plasma drug concentration decayed with a biexponen-
a central and a peripheral compartment, with elimination from the central compartment. Pharmacokinetic calculations with this model (Table 1) indicated an elimination half-life of 52 min in control rats. Pretreatment with wild-type BChE, 1 mg/kg, caused a slight increase in levels of the cocaine breakdown product, benzoic acid, but no change in cocaine’s pharmacokinetic parameters, including elimination half-life and AUC. This result was anticipated because of the relatively small enzyme dosage. An identical dose of A328W/Y332A, however, accelerated cocaine metabolism markedly (Fig. 3A). With this pretreatment, drug levels in early plasma samples were 40% below control and continued to drop steeply with time. Pharmacokinetic calculations (Table 1) showed a halving of AUC, a 34% reduction of elimination half-life, and a doubling of cocaine clearance. Accompanying these changes was a 10-fold rise in levels of benzoic acid (Fig. 3B). Even larger effects were generated by a 3 mg/kg dose of A328W/Y332A (Fig. 3). In fact, cocaine clearance, t_{1/2e}, and AUC all showed a significant and near-linear dependence on BChE dosage (Fig. 4).

LC-MS measurements validated the radiometric data. First, LC-MS showed that the toluene-extracted radioactivity ascribed to cocaine was not attributable to benzoylecgonine or norcocaine. Although those metabolites would have retained the [3H]benzoyl label from [3H]cocaine, they were below the limit of LC-MS detection in our “cocaine fractions” (toluene extracts from alkaline buffer). Likewise, cocaine and other metabolites were not detected by LC-MS in the “benzoic acid fractions” (extracts from acidic buffer). Thus, the results in Table 1 accurately reflect the in vivo conversion of cocaine to benzoic acid.

LC-MS also provided an additional insight into the metabolic pathways for cocaine disposal under our experimental conditions. Using this method, cocaine, benzoylecgonine, norcocaine, and ecgonine methyl ester were analyzed at 15, 30, and 60 min after the i.v. injection of cocaine (Fig. 5). In control rats, plasma cocaine reached high levels immediately after the injection and were still detectable at 60 min. EME, on the other hand, was barely detectable at any time. Since BChE-catalyzed hydrolysis produces EME along with benzoic acid (not measured by our LC-MS procedure), it appears that endogenous BChE did not contribute much to cocaine metabolism. LC-MS also failed to detect significant levels of norcocaine, a product of the cytochrome P450 system. Benzoylecgonine, on the other hand, accumulated to a measurable extent, indicating that carboxylesterase played a metabolic role. In rats pretreated with A328W/Y332A, norcocaine was also not detected and benzoylecgonine also accumulated, albeit slowly. In these animals, however, plasma cocaine was nearly eliminated at 15 min after the injection, whereas EME rose to a level nearly 8 times higher than that of benzoylecgonine. Thus, in this experimental group, hydrolysis by BChE became the major pathway for cocaine metabolism.

**Pharmacokinetics of Intraperitoneal Cocaine.** Returning to the radiometric approach, for ease of quantitation, we attempted to determine whether the effects of A328W/Y332A might be influenced by the route of cocaine administration. Specifically, in rats challenged with cocaine in an i.p. dose of 60 mg/kg, we examined the effects of BChE pretreat-

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**TABLE 1**

Pharmacokinetics of intravenous cocaine

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Wild-Type BChE (1 mg/kg)</th>
<th>A328W/Y332A (1 mg/kg)</th>
<th>A328W/Y332A (3 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t_{1/2e} (min)</td>
<td>2.9 ± 0.25</td>
<td>3.0 ± 0.13</td>
<td>1.6 ± 0.17**</td>
</tr>
<tr>
<td></td>
<td>AUC (min · μM)</td>
<td>160 ± 3.6</td>
<td>156 ± 8.0</td>
<td>86 ± 6.1***</td>
</tr>
<tr>
<td></td>
<td>CL (l/min/kg)</td>
<td>0.13 ± 0.003</td>
<td>0.13 ± 0.007</td>
<td>0.24 ± 0.02***</td>
</tr>
<tr>
<td></td>
<td>MRT (min)</td>
<td>64 ± 4.0</td>
<td>69 ± 4.9</td>
<td>54 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>V_{area} (l/kg)</td>
<td>8.0 ± 0.40</td>
<td>8.8 ± 0.31</td>
<td>13 ± 1.8*</td>
</tr>
</tbody>
</table>

Asterisks indicate significant differences from control: *p < 0.05, **p < 0.01, ***p < 0.001.
ment on elimination half-life, AUC, absorption half-life, peak plasma concentration, and time to peak. Absorption half-life and time to peak remained stable under all tested conditions (Table 2, Fig. 6). In contrast, elimination half-life, AUC, peak plasma concentration, and benzoic acid levels depended on the type of pretreatment. Wild-type BChE in a dose of 1 mg/kg had no effect, but the same dose of A328W/Y332A BChE enhanced all these measures of cocaine disposal. It is worth stressing that the experiments with i.p and i.v. cocaine were not only performed independently but also were analyzed by different pharmacokinetic models (see Experimental Procedures). For this reason, the close agreement between pharmacokinetic parameters in Tables 1 and 2 is remarkable. The combined data show convincingly that a modest dose of A328W/Y332A can accelerate cocaine disposal to an extent that should be clinically significant.

**Discussion**

**Assay and Properties of Cocaine Hydrolase.** We have used a simple radiometric assay validated by LC-MS to demonstrate cocaine removal by an effective hydrolase and to characterize the pharmacokinetics. The ability to assay blood samples as small as 100 μl enabled repeated measurements without hemodynamic disturbance. A second prerequisite for these experiments was a recombinant BChE that would continue to function for 1 or 2 h after an i.v. injection. Native human plasma BChE is a tetrameric enzyme with a plasma half-life of about 24 h after injection into rats (Lynch et al., 1997; Mattes et al., 1997), but recombinant BChE is typically much less stable. When prepared by ordinary methods, recombinant BChE is mainly monomeric or dimeric and, for some reason, has a very short residence time in the blood circulation, often disappearing within minutes (Saxena et al., 1998). Since recombinant tetrabmers are more stable, we prepared a mostly tetrameric A328W/Y332A BChE by cotransfection with COLQ cDNA. In line with previous observations

**TABLE 2**

Pharmacokinetics of intraperitoneal cocaine

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Wild-Type BChE (1 mg/kg)</th>
<th>A328W/Y332A BChE (1 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t₁/₂abs (min)</td>
<td>53 ± 1.6</td>
<td>50 ± 7.3</td>
<td>34 ± 3.2**</td>
</tr>
<tr>
<td>t₁/₂el (min)</td>
<td>3.7 ± 0.3</td>
<td>3.1 ± 0.3</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>AUC (min ⋅ μM)</td>
<td>160 ± 15</td>
<td>160 ± 12</td>
<td>80 ± 3.3**</td>
</tr>
<tr>
<td>Tₘ₉₅ (min)</td>
<td>13 ± 2.0</td>
<td>13 ± 1.1</td>
<td>15 ± 1.1</td>
</tr>
<tr>
<td>Cₘ₉₅ (μM)</td>
<td>1.8 ± 0.15</td>
<td>1.7 ± 0.15</td>
<td>1.1 ± 0.1**</td>
</tr>
</tbody>
</table>

Asterisks indicate significant differences from control: **p < 0.01, ***p < 0.001.
in mice (O. Lockridge, unpublished data), the resulting enzyme had a half-life in excess of 9 h in rats. This BChE exhibited an unexpectedly large volume of distribution, however, as if there had been significant transfer out of the vascular system. Native BChE, being a sizeable protein (monomer molecular mass, 85 kDa), does not readily leave the circulation (Mattes et al., 1997). One explanation for the large apparent volume of distribution, therefore, is that the injected BChE contained appreciable amounts of short-lived forms that were rapidly cleared from plasma. Nonetheless, the demonstrated average stability should be ample for cocaine detoxification in a clinical setting.

**Pharmacokinetics of Cocaine.** Our baseline data agree well in general with the literature on uptake, distribution, and elimination of cocaine in many species, including rats (Barber et al., 1992; Pan and Heady, 1997; Barat and Abdel-Rahman, 1998; Lau et al., 1999). A consistent finding with cocaine is rapid redistribution from plasma into a theoretical volume that exceeds total body water, probably because the drug accumulates in lipid-rich tissues such as the brain. In our hands, regardless of injection route, the observed peak levels of plasma cocaine were lower than would be expected if the drug had mixed instantaneously with total body water. We are confident that this feature does not reflect aberrant behavior of radiolabeled cocaine, such as selective retention at the site of injection. In the first place, a pilot experiment with samples taken 15 min after tail vein injection recovered less than 5% of the total injected radioactivity in digested tail tissue (not shown). In the second place, almost identical peak values were reported recently by others using HPLC methods to characterize the pharmacokinetics of cocaine after i.v. injection in the rat (Pan and Hedaya, 1999; Sun and Lau, 2001). Given the rapidity of the redistribution of cocaine, we did not expect the apparent 50% shortening of $t_{1/2}$, after treatment with A328W/Y332A (Table 1). This outcome may merely reflect the limitations of bi-exponential curve fitting when elimination is fast enough to overlap with redistribution.

The elimination half-life of cocaine in rats has been estimated to range from 15 to 90 min, but values near 30 min are typical (Misra et al., 1977; Pan and Hedaya, 1997; Barat and Abdel-Rahman, 1998; Lau et al., 1999). Our observed half-life was above average, perhaps owing to the urethane anesthesia, which can reduce cardiac output and blood flow to metabolic sites in the liver. Much of the cocaine elimination in control animals may have been driven by carboxylesterase, which rats express in plasma as well as liver. BChE normally contributes little to cocaine metabolism in rats (Stewart et al., 1978). For one thing, this enzyme is only 5% as abundant in rat plasma as in human plasma (Li et al., 2000); for another, rat BChE is a relatively poor cocaine hydrolase (O. Lockridge, unpublished data). Thus, it is not surprising that plasma levels of the BChE-related metabolites, benzoic acid and EME, remained low when control rats were challenged with cocaine. But the marked effects of A328W/Y332A show that an effective cocaine hydrolase can substantially accelerate cocaine elimination and shift the metabolic profile so that BChE-generated metabolites become prominent.

Our negative findings with wild-type human BChE are consistent with previous results in cats given a 10-fold larger dose of the same enzyme (Mattes et al., 1997). In that study, the elimination half-life of cocaine was not shortened; the only observable effects were a slight reduction in peak level of plasma cocaine and a slight increase of EME. A more recent study with primates given a similarly large dose of horse BChE (Carmona et al., 2000) reported 3-fold reduction of peak cocaine concentration (at 1 min) but relatively modest changes in the pattern of cocaine metabolites. Given the possibility of postsampling hydrolysis in samples with high concentrations of BChE, it can even be questioned whether the reduction in peak plasma levels was genuine. We found it necessary to include iso-OMPA and echotriphosphate along with sodium fluoride to eliminate that potential source of artifact. Altogether, these results show that the ability of wild-type BChE to accelerate cocaine metabolism in vivo is limited, although, when sufficient amounts are administered, significant effects emerge.

**A328W/Y332A for Cocaine Detoxication.** Normal levels of endogenous BChE are saturated immediately by bolus administration of a stoichiometric excess of cocaine, as in the pattern of binging among cocaine abusers. Administered in large amounts, wild-type human BChE can reduce cocaine levels in plasma and important target organs and appears to confer some protection against cocaine toxicity (Mattes et al., 1997). Our results suggest that smaller amounts of the catalytically improved hydrolase, A328W/Y332A, will be able to confer equivalent or greater protection. In particular, the finding of reduced cocaine levels in brain and heart after treatment with this enzyme (Sun et al., 2001, 2002) suggest a potential role in combating cocaine-induced seizures and cardiac arrhythmias.

In the present experiments, A328W/Y332A was given as a pretreatment, before cocaine challenge. Although the data indicate potential therapeutic value and offer “proof of concept,” it remains to be seen how effectively the enzyme will rescue rats from cocaine toxicity when administered after the fact. It is still less certain that any cocaine hydrolase will function against cocaine overdoses presenting in hospital emergency rooms, often well after the initial intoxication. However, earlier results indicating that inefficient wild-type BChE can rescue mice and rats (Hoffman et al., 1996; Lynch et al., 1997) justify optimism that improved cocaine hydrolases will be useful in the clinical setting. Studies are ongoing to evaluate the effects of this A328W/Y332A on the physiologic and toxicologic responses to cocaine and to explore potential uses in treating cocaine toxicity.

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