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Gene Transfer of Cocaine Hydrolase Suppresses Cardiovascular Responses to Cocaine in Rats

Yang Gao, Elena Atanasova, Nan Sui, James D. Pancook, Jeffry D. Watkins, and Stephen Brimijoin

Department of Molecular Pharmacology, Mayo Clinic, Rochester, Minnesota (Y.G., E.A., N.S., S.B.); Eli Lilly-Applied Molecular Evolution, San Diego, California (J.D.P., J.D.W.); and Institute of Psychology, Chinese Academy of Science, Beijing, China (N.S.)

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ABSTRACT

We previously found that injection of a cocaine hydrolase (CocE) engineered from human butyrylcholinesterase will transiently accelerate cocaine metabolism in rats while reducing physiological and behavioral responses. To investigate more extended therapeutic effects, CocE cDNA was incorporated into a replication-incompetent type-5 adenoviral vector with a cytomegalovirus promoter. In rats dosed with this agent (2.2×10^9 plaque-forming units), the time course of expression was characterized by reverse transcription polymerase chain reaction for CocE mRNA and by radiometric assay for enzyme activity. Liver and plasma showed comparable expression, beginning 2 days after vector administration and peaking between 5 and 7 days. Plasma CocE content was up to 100 mU/ml, with total cocaine hydrolyzing activity 3000-fold greater than in "empty vector" or untreated controls. This level of expression approximated that found immediately after i.v. injection of purified hydrolase, 3 mg/kg, a dose that shortened cocaine half-life and blunted cardiovascular effects. Sucrose density gradient analysis showed that 96% of the circulating CocE activity was associated with tetrameric enzyme forms, expected to be stable in vivo. Consistent with this expectation, CocE from vector-treated rats showed a plasma $t_{1/2}$ of 33 h when reinjected into naive rats. Transduction of another mutant butyrylcholinesterase, Applied Molecular Evolution mutant 359 (AME_{359}), caused plasma cocaine hydrolase activity to rise 50,000-fold. At the point of peak AME_{359} expression, cocaine was cleared from the blood too rapidly for accurate measurement, and pressor responses to the injection of drug were greatly impaired.

Abuse of cocaine, one of the most addictive substances known, is a continuing medical, social, and political problem. Effective therapies for cocaine abuse do not exist today, and new strategies are needed (Benowitz, 1993; Carroll et al., 1999). In the central nervous system cocaine enhances neurotransmitters involved in pleasure and reward, including norepinephrine, dopamine, and serotonin (Johanson and Fischman, 1989). This enhancement reflects the blockade of multiple membrane transporter proteins for biogenic amines (Ritz et al., 1987; Amara and Kuhar, 1993), a mode of action that makes it difficult to design "cocaine antagonists". However, we can try to diminish cocaine's psychoactive and addictive potency by increasing its metabolic clearance.

Two cocaine hydrolases have recently been engineered from human plasma cholinesterase (Sun et al., 2001, 2002a; Pancook et al., 2003). One, which we designate CocE, exhibits several therapeutically promising properties in rodents: rapid removal of cocaine from the bloodstream and reduced accumulation in nervous tissue (Sun et al., 2002b); reversal of cardiovascular responses to cocaine without exertion of direct physiological effects (Gao and Brimijoin, 2004); and blockade of cocaine-induced hyperactivity (Sun et al., 2002a). In preliminary tests, a newer hydrolase designated AME_{359} has been even more effective (J. Pancook, unpublished data). Sustained delivery of such enzymes might limit cocaine's access to targets in the central nervous system and reduce its reward potential. If that goal could be achieved without deleterious side effects, the hydrolases could be tried in drug cessation programs for cocaine addiction. We have now evaluated the feasibility of modifying cocaine responsiveness in rats for days to weeks by gene therapy with adenoviral vec-

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ABBREVIATIONS: AME_{359}, applied molecular evolution mutant 359; Ad5, type 5 adenoviral vector; CMV, cytomegalovirus promoter; CocE, A328W/Y332A rQ45 butyrylcholinesterase; BChE, butyrylcholinesterase; DFP, diisopropyl-fluorophosphate; PFU, plaque-forming unit(s); RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; bp, base pair(s).

tors. Here, we report the time course and location of virally transduced cocaine hydrolases, their effects on cocaine metabolism, and deposition in heart and brain, and, awaiting future behavioral studies, their ability to blunt cardiovascular responses.

Materials and Methods

Materials. CocE is a doubly mutated version of human BChE (A328W/Y332A rQ45) engineered by Sun et al. (2001). Recombinant CocE was obtained from Chinese hamster ovary K1 cells cotransfected with truncated rat ColQ to promote stable tetrameric forms (Krejci et al., 1997; Altamirano and Lockridge, 1999; Xie et al., 1999). Product purified by affinity chromatography on procainamide-Sepharose was generously supplied by Dr. Oksana Lockridge (University of Nebraska, Lincoln, NE). Murine monoclonal anti-human BChE (B12) was drawn from stocks originally produced in our laboratory (Brimijoin et al., 1983). Natural (−)-cocaine hydrochloride, atropine sulfate, heparin sulfate, NaF, rabbit anti-rat IgG and anti-rat IgM, tetraisopropyl pyrophosphoramide, diisopropyl-fluorophosphate (DFP), and urethane anesthetic were all obtained from Sigma-Aldrich (St. Louis, MO). [^{3}H](−)-Cocaine was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Pansorbin was from Calbiochem-Behring (Temecula, CA). The agent (2-mercaptoethyl) trimethylammonium iodide *O*,*O*-diethyl phosphorothioate (echothiophate iodide) was from Wyeth-Ayerst (Philadelphia, PA). T4 DNA-ligase, BamHI, and HindIII were from Roche Diagnostics (Mannheim, Germany).

Construction of Adenoviral Vectors. Type-5 replication-incompetent adenoviral vectors with cytomegalovirus promoter (Ad5-CMV) were chosen for ability to accommodate our enzyme cDNAs and transduce gene product efficiently in vivo (Thomas et al., 2003). We created expression cassettes with CocE and AME_{359}, a human BChE with four mutations: A328W, Y332M, S287G, and F227A (Pancook et al., 2003). Each cDNA was digested with BamHI and HindIII (overnight, 37°C) and ligated with T4 ligase (overnight, 14°C) into a similarly digested shuttle plasmid containing promoter and flanking sequences of adenoviral DNA. Recombinant DNA was then transformed and amplified in *Escherichia coli.* The final vectors, Ad5-CMV-CocE and Ad5-CMV-AME_{359}, were assembled in the Gene Transfer Core Facility at the University of Iowa (Iowa City, IA; Dr. Beverly Davidson, director). Assembled vectors were evaluated before use in live rats by transduction in human umbilical vein endothelial cells (90% confluent cultures in 60-mm dishes). Two days after exposure to 2.5×10^8 plaque-forming units (PFU), cocaine hydrolase activity in culture supernatants rose more than 1000-fold. Parallel assays detected no activity in the vector stock solution.

Preparation of Animals. Animal studies, conducted under a protocol approved by the Mayo Institutional Animal Care and Use Committee, used male Sprague-Dawley rats weighing 250 to 350 g (Harlan, Madison, WI). To transduce cocaine hydrolase, a single injection of viral vector was given through the tail vein, 2.2 to 11×10^9 PFU. Animals were monitored for up to 3 weeks and small blood samples (<0.3 ml) were collected every other day for the first week, and less frequently thereafter.

For continuous monitoring of blood pressure the rats were anesthetized with urethane (1.3 g/kg i.p.). A polyethylene-50 cannula was then placed in the femoral artery on one side and connected to a pressure transducer (Gould TA240). Another cannula was placed in the ipsilateral femoral vein for blood sampling, and an infusion set was introduced into the lateral tail vein for drug administration. 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.

Drug Administration. All drugs were prepared in 0.9% NaCl and were administered through the lateral tail vein. Atropine sulfate, 1 mg/kg, was injected before cocaine challenge, to block vagal reflexes that ordinarily limit drug-induced pressor responses (Gao and Brimijoin, 2004). Ten minutes later, (−)-cocaine hydrochloride, 3.5 mg/kg, was infused over a period of 10 s (total volume, 5 ml/kg). Blood pressure was recorded continuously during this infusion and for 10 min thereafter.

Blood and Tissue Collection. To assess expression levels of virally transduced enzyme, blood samples were drawn from the tail vein, collected in heparinized tubes, and centrifuged at once to obtain plasma, which was stored at −80°C. The rats were then perfused through the aorta with 250 to 350 ml of 0.9% NaCl, and representative tissues were frozen at −80°C for later enzyme assay (completed within 24 h). To determine the fate of [^{3}H]cocaine after i.v. injection, miniature blood samples (100–200 μl) were drawn repeatedly from the femoral vein (less than 400 μl in all during the 10 min when blood pressure was monitored). Blood samples were collected in heparinized tubes containing BChE inhibitors (echothiophate and tetraisopropyl pyrophosphoramide, 10^{-5} M final concentration, and NaF diluted 40-fold from a saturated solution). The same BChE inhibitors were added to the saline perfusion medium before collecting tissues for [^{3}H]cocaine determination.

Detection of mRNA for CocE. For RT-PCR analysis, mRNA was extracted from tissues using a FastTrak 2.0 kit (Invitrogen, Carlsbad, CA). Reverse transcription was carried out with 1 μg of mRNA using a Superscript II first-strand synthesis kit (Invitrogen) and random hexamer primers; 25-ng aliquots of the resulting cDNA were used for subsequent PCR amplification. An 828-bp fragment of rat BChE cDNA was amplified with the following primers: sense (5′ position 808, GenBank accession no. AF244349), AGA GCC ATT CTA GAA AGT GG; and antisense (5′ position 1636), TTG TGA AGA CAG GCC ACA. To amplify a 294-bp fragment of CocE with no endogenous signal, two primers for normal human BChE were designed with 3′ ends containing multiple mismatches to the rat sequence: sense (5′ position 1055, accession no. M16541), CCT ATG GGA CTC CTT TG; and antisense (5′ position 1349), TCA TCT ACC CAG TCT GTG. These primers amplified no DNA in mRNA preparations from naive rats but with transduced rats they amplified a fragment spanning the two CocE mutations. As an assay control, a 508-bp fragment of rat cyclophilin was amplified with the following primers: sense (5′ position 49, accession no. M19533), AAC CCC ACC GTG TTC TTC; and antisense (5′ position 557), ATG CCC GCA AGT CAA AGA.

RT-PCR reactions were performed in 50-μl volumes with the Hotstart *Taq* Master Mix (QIAGEN, Valencia, CA) using 15 min at 95° for *Taq* activation. Samples were subjected to 35 cycles of melting (30 s at 95°), annealing (20 s at 50°), and extension (30 s at 72°), with final extension for 10 min at 72°. Reactions were carried out in 1× PCR buffer containing 1.5 mM $MgCl_2$, according to the manufacturers instructions. Specific DNA amplicons excised from agarose gels were extracted by the Wizard Purification System (Promega, Madison, WI). Bidirectional sequencing was performed in the Mayo Molecular Biology Core Facility by the *Taq*-dideoxy dye terminator cycle method with sequence-specific primers. Results of this analysis confirmed the product identities.

Radiometric Assays. 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 10,000*g* for 10 min. To evaluate cocaine hydrolase activity in plasma and tissue samples, we used a radiometric assay based on release of [^{3}H]benzoic acid from [^{3}H]cocaine followed by selective partitioning from acid medium into toluene-based fluor for liquid scintillation counting (Brimijoin et al., 2002). Assays ran at a final cocaine concentration of 18 μM, the K_m value for CocE, and data were expressed in nanomoles of product per minute (milliunits) after extrapolation to V_{max}. Related assay procedures were used to determine plasma and tissue concentrations of [^{3}H]cocaine and [^{3}H]benzoic acid as described in detail previously (Sun et al., 2002b; Gao and Brimijoin, 2004).

Immunoadsorption Assays. Solid-phase immunoadsorption was sometimes used to separate transduced CocE from endogenous rat BChE. For this purpose, killed *Staphylococcus aureus* cells (Pansorbin) were coated with rabbit anti-mouse IgG followed by murine monoclonal antibodies selective for human BChE (final concentration $\approx 10^{-7}$ M). Plasma samples or tissue extracts were incubated with immunoadsorbent for 1 h at 37°C. After centrifugation (2000*g*, 10 min), cocaine hydrolase activity in supernatant fractions and pellets (rinsed three times with original volume of buffer) was assayed as described above. A modified version of the immunoadsorption system was also used to search for circulating antibodies against transduced CocE. In this case, Pansorbin aliquots saturated with linker antibodies against rat IgG or IgM were exposed for 1 h at 37°C to 100-μl samples of rat plasma plus 150 μl of 50 mM sodium phosphate, pH 7.4, followed by three cycles of buffer washes and centrifugation at 2000*g* for 10 min. These preparations, with adsorbed antibody if any, were then tested for their ability to bind CocE during a 1-h exposure to a standard sample of transduced plasma, using enzyme activity of supernatant and pellet fractions as a guide.

Sucrose Density Gradient Fractionation. Molecular forms of hydrolases 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 Inc., Fullerton, CA). Numbered fractions (200 μl) recovered from the bottoms of the tubes were assayed for cocaine hydrolase activity.

Statistical Analysis and Pharmacokinetics. Student's t test was used for statistical analysis of group differences in enzyme activity. 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. Concentration-time profiles of plasma enzyme activity were analyzed with Sigma Plot 4.1 (SPSS Inc., Chicago, IL). Data from virally transduced rats were fitted to a simple exponential decay model: $\text{CocE} = \text{Ae}^{-\alpha t}$. Biphasic data obtained after injection of purified CocE were fitted to the equation $\text{CocE} = \text{Ae}^{-\alpha t} + \text{Be}^{-\beta t}$.

Results

Time Course and Tissue Expression. To track CocE transduction, we assayed the enzyme in plasma sampled over 3 weeks from four rats that received 2.2×10^9 PFU of Ad5-CMV-CocE vector on day 0 (Fig. 1). On gross inspection these animals seemed in good health. Their plasma CocE activity began rising steeply on day 2, reached a peak at days 5 to 7, and then declined sharply. Even at 21 days, however, the activity remained 5-fold above pretreatment levels. By contrast, cocaine hydrolase activity was barely detectable in untreated controls and rats treated with "empty vector" lacking the CocE expression cassette, reflecting the low efficiency of endogenous rat BChE in this reaction.

We used selective immunoadsorption to confirm that virally driven CocE expression was directly responsible for the cocaine hydrolase activity measured after vector treatment. Adsorption was performed with B-12, a murine monoclonal antibody previously shown to exhibit high affinity for native human BChE but no measurable affinity for rat BChE (Brimijoin et al., 1983). Incubation for 1 h with solid phase B-12 removed 99% of the cocaine hydrolase activity in plasma drawn 5 days after administration of Ad5-CMV-CocE. We concluded that this activity represented transduced enzyme that was recognizable to an anti-human BChE antibody, despite having been generated in the rat. In support, the kinetic behavior of this enzyme was very close to that of purified recombinant CocE (mean cocaine K_m value in plasma samples from two rats was 17 μM).

The tissue distribution of CocE was examined in four other rats treated 5 days earlier with Ad5-CMV-CocE (Fig. 2). Identical patterns were seen in whole tissue homogenates and in extracts adsorbed to B-12 antibody (data not shown). All seven tissues tested in control rats showed trivial levels of baseline cocaine hydrolase activity (<0.025 mU/g). After viral transduction, however, activity in liver was 45 ± 3 times greater than in rats treated with empty vector (which were equivalent to untreated rats). Other tissues showed very small increases in the absolute amount of hydrolase activity, although the 2- to 3-fold changes in diaphragm, heart, and spleen were statistically significant. Brain and rinsed erythrocytes displayed virtually no activity before or after transduction.

The foregoing data suggested that liver was the primary source of transduced CocE in plasma. This conclusion was supported by RT-PCR analysis of mRNA extracted from a set of tissues sampled 5 and 9 days after vector injection, which yielded CocE cDNA amplicons only in liver and only at the 5-day point (Fig. 3). In light of this result, the time course of enzyme expression in liver was followed in two rats. The data showed that, after rising steadily to a peak on day 5, cocaine hydrolase activity largely disappeared from liver by day 7 (Fig. 4). This finding, consistent with the failure to detect CocE mRNA at 9 days, led us to investigate the stability and half-life of the transduced CocE circulating in plasma.

Stability of Transduced CocE. CocE half-life was assessed directly in the following way. Plasma from six transduced rats (5 days after exposure to Ad5-CMV-CocE, 2.2×10^9 PFU) was collected and injected into an equal number of naive recipient rats. Circulating cocaine hydrolase activity in the recipients was observed to decline in a simple exponential manner with a half-life ($t_{1/2}$) of 33 ± 2.6 h (Fig. 5). Three experiments with transduced AME_{359} indicated a similar pattern of decay with a slightly shorter $t_{1/2}$ of 27 ± 1.7 h (not shown). This result contrasted with the behavior of recombinant CocE purified from Chinese hamster ovary cells, which

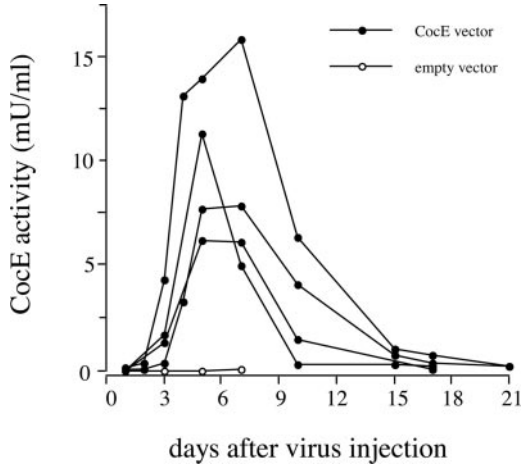

Fig. 1. Time course of CocE expression in plasma. Cocaine hydrolase activity was determined in blood samples taken from the tail vein on days 1 to 21 after rats were transduced with 2.2×10^9 PFU of Ad5-CMV-CocE (filled circles) or empty Ad5-CMV vector (open circles). Data are values from individual rats.

decayed in biexponential manner with an initial $t_{1/2}$ of 40 min and a terminal $t_{1/2}$ of 12 h.

We tested the possibility that the persistence of CocE in passive recipients might have reflected ongoing transduction of new enzyme by vector surviving in the donor plasma. This possibility was eliminated by an experiment in which donor CocE was inactivated by 1-h treatment with the irreversible anticholinesterase DFP (10^{-5} M), followed by dialysis overnight. Injection of such treated plasma into another rat yielded no measurable cocaine hydrolase activity at any time between 1 and 120 h. On the other hand, DFP treatment of the stock Ad5-CMV-CocE vector did not diminish transduction capacity in two further rats (data not shown). Together, these observations indicate that a long natural-half-life accounts for the slow decay of activity after reinjection of transduced CocE.

BChE stability is influenced by oligomeric state. Therefore, ultracentrifugation on sucrose density gradients was used to compare the molecular forms of recombinant and virally transduced enzymes (Fig. 6). Recombinant CocE and trans-

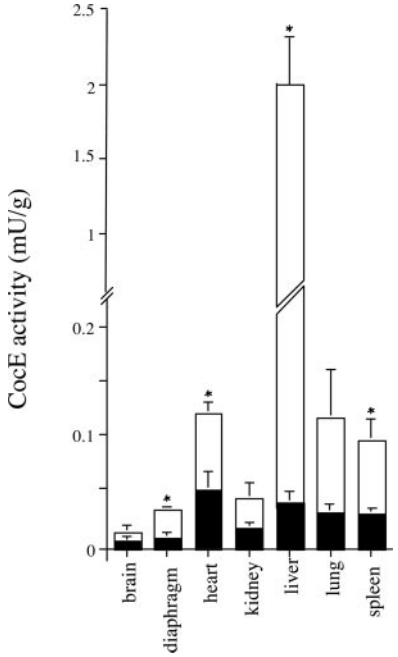

Fig. 2. CocE distribution in tissues before and after viral transduction. White columns are cocaine hydrolase activity in four rats euthanized 5 days after transduction with 2.2×10^9 PFU Ad5-CMV-CocE. Black columns are activity in four untreated control rats. Tissues were collected after 250 to 350 ml of 0.9% NaCl was perfused through the aorta. Means ± S.E.M. are shown. Note the break and shift in scale to accommodate the large values from liver. Asterisks indicate statistically significant increases after transduction ($p < 0.05$).

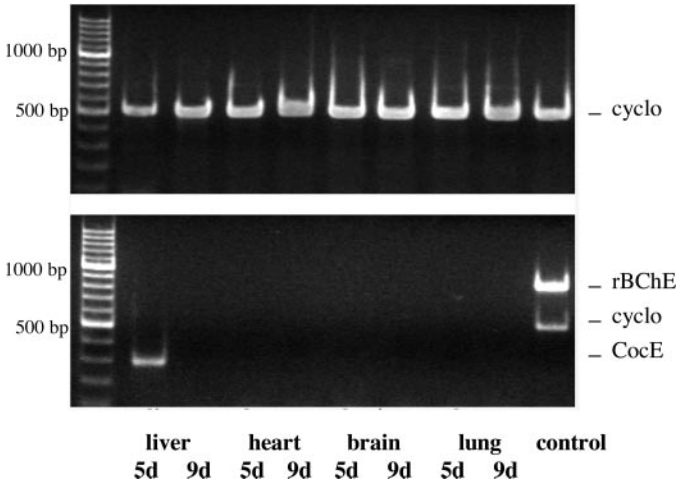

Fig. 3. CocE mRNA detected by RT-PCR. At 5 and 9 days after transduction with 2.2×10^9 PFU of Ad5-CMV-CocE, mRNA was isolated from tissues and subjected to PCR amplification (see *Materials and Methods*). Top, loading and transcription controls showing uniform expression levels of cyclophilin (cyclo). Bottom, specific amplification of CocE, and, in a control reaction with liver, rat BChE (rBChE) and cyclophilin. The CocE amplicon in liver at 5 days, the rBChE amplicon in control liver, and the cyclophilin amplicons in all samples are of the expected sizes (294, 828, and 528 bp); their identities were confirmed by dideoxy sequencing.

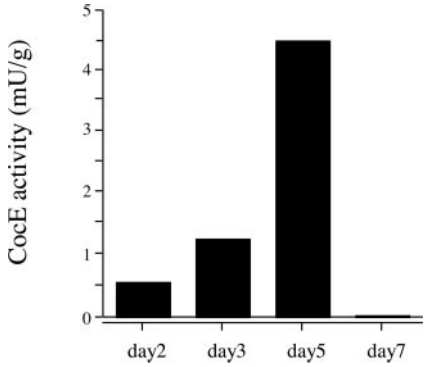

Fig. 4. Time course of CocE expression in liver. Eight rats received 2.2×10^9 PFU Ad5-CMV-CocE at day 0. Liver samples were collected from two rats at each time point after NaCl perfusion. Mean levels of cocaine hydrolase activity are shown.

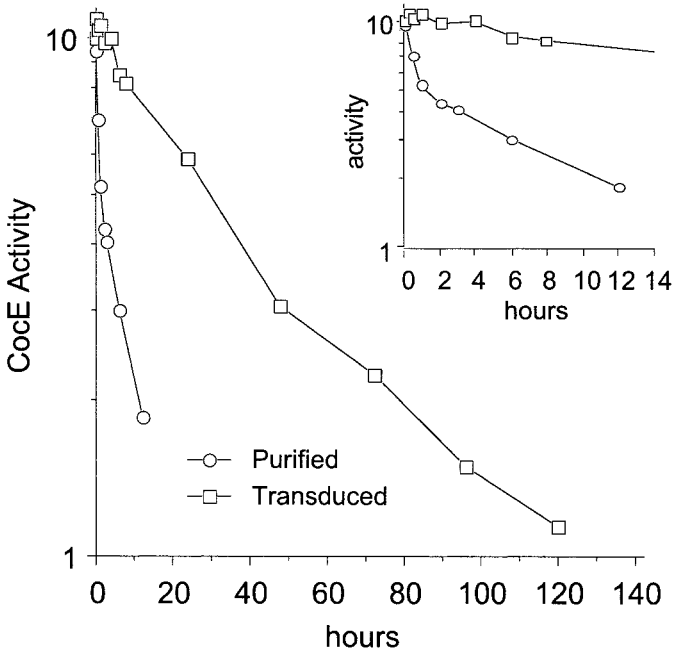

Fig. 5. Stability of transduced CocE in vivo. At time 0, plasma from rats transduced with Ad5-CMV-CocE (11×10^9 PFU, 5 days before) was injected into the tail veins of six naive recipient rats. At the indicated times blood samples were taken from these rats and assayed for cocaine hydrolase activity (open squares). Data (means ± S.E.M.) were normalized to match starting activities from rats sampled previously after injection of purified recombinant CocE (open circles, data from Sun et al., 2002). Actual mean activity in the first sample was 6 mU/ml (10-fold lower than in the donor plasma). Inset, same data plotted on a shorter time scale. Note biphasic decay of recombinant CocE and monophasic decay of transduced CocE.

duced AME_{359} both contained large amounts of the stable tetrameric form but also a substantial fraction of low molecular weight enzyme. Transduced CocE, in contrast, was entirely tetrameric, like native plasma BChE of humans and rats.

Because CocE was transduced as a highly stable tetramer it was surprising that plasma cocaine hydrolase activity dropped so rapidly after 1 week. To determine whether immune reactions might play a destabilizing role, we investigated one rat that had shown a particularly sharp loss after day 5, dropping to 0.2% of peak on day 9. That same day, 4 min after this rat received 1 ml of plasma from a day 5 donor, cocaine hydrolase activity rose only to 10% of the expected level. The activity then fell 85% in 1 h, and it became undetectable by 6 h (data not shown).

The preceding results indicate that CocE clearance rose greatly during the second week after transduction. One reasonable mechanism for accelerated removal would be complex formation with antibodies against this human-derived protein. However, we were unable to detect circulating IgG or IgM antibodies to CocE. More specifically, after incubating plasma sampled between days 1 and 21 with solid-phase, anti-rat IgG or IgM, we found no removal of CocE activity from the supernatants and no measurable CocE activity in the pellets.

Accelerated Cocaine Metabolism after Transduction of Hydrolases. Before evaluating effects on cocaine's pharmacokinetics and pharmacodynamics, the dose-response relations of viral transduction were examined. Raising the dose of Ad5-CMV-CocE by factors of 3, 5, and 10 caused progressively greater transduction of plasma CocE (Fig. 7). Mean cocaine hydrolase activity 5 days after transduction with the highest dose was 50 ± 8 mU/ml, equivalent to 27 μg/ml of purified protein. That value represents a 3000-fold increase above baseline. Still greater cocaine hydrolase activity, averaging 780 ± 400 mU/ml, or 50,000-fold above baseline, was seen after transduction with an equivalent dose of Ad5-CMV-AME_{359}.

To determine whether a transduced hydrolase would accelerate cocaine metabolism, we began with an in vitro approach. Cocaine in a final concentration of 37 μM was added to plasma from one control rat and one rat transduced 5 days earlier with Ad5-CMV-CocE (11×10^9 PFU). These samples contained, respectively, 0.05 and 100 mU of CocE/ml. Although cocaine was extremely stable in the control plasma, it disappeared from the transduced plasma within an hour, replaced by near stoichiometric amounts of the metabolite benzoic acid (Fig. 8).

The metabolic effect of transduced CocE was somewhat weaker in vivo. On average, rats transduced with 11×10^9 PFU of vector expressed only half as much CocE as the donor for the in vitro experiment. This level of expression scarcely affected the rate of cocaine elimination after i.v. injection, although it significantly reduced the amount of drug in the initial sample (Fig. 9). Cocaine elimination was faster in individual rats with the highest levels of CocE activity, however, and was extremely rapid in animals transduced with the still more efficient hydrolase AME_{359}. Plasma from AME-transduced rats sampled 4 min after drug injection contained 95% less cocaine than comparable samples from control rats. At the same time, these animals showed marked reductions of cocaine in brain (55%) and heart (85%), together with benzoic acid levels that were far above normal. These pharmacokinetic effects warranted a study of physiological responses in rats expressing exogenous hydrolases.

Physiological Impact of Transduced Hydrolases. Rats given high doses of vector (11×10^9 PFU) remained normal in general appearance, motor activity, and body weight, although some showed yellow plasma. We examined pressor responses to cocaine 5 days after high-dose transduction. Four groups were studied: untreated (n = 13), empty vector (n = 5), Ad5-CMV-CocE (n = 8), and Ad5-CMV-AME_{359} (n = 5). The rats were anesthetized with urethane and instrumented for continuous recording of blood pressure in the femoral artery. After stabilization, all animals were pretreated with atropine to prolong their pressor response (see *Materials and Methods*). Ten minutes later, they were challenged with i.v. cocaine, 3.5 mg/kg. Because cocaine caused the same effects in rats given empty vector as in untreated rats (Fig. 10A), data from these animals were pooled. The combined results demonstrated a statistically significant reduction of pressure in the CocE group and a more substantial effect in the AME_{359} group (Fig. 10B). The reduction in both cases was apparent within 60 s after co-

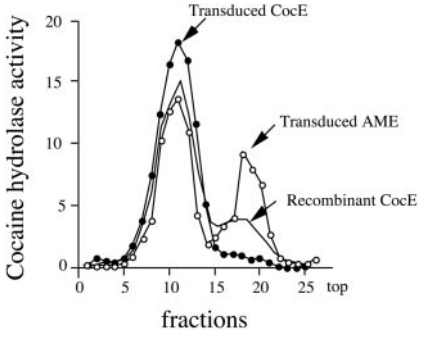

Fig. 6. Sucrose density gradient analysis of hydrolase forms in plasma. Filled and open circles are data from rats 5 days after transduction with 2.2×10^9 PFU of Ad5-CMV-CocE (n = 2) or Ad5-CMV-AME_{359} (n = 1), respectively. The simple line indicates activities in plasma collected from a single rat, 2 h after injection of purified recombinant enzyme, 3 mg/kg. Activities are means of duplicate assays, expressed in arbitrary units. Top of gradient is toward the right. Fractions 9 to 12 correspond to the G4 or tetrameric form.

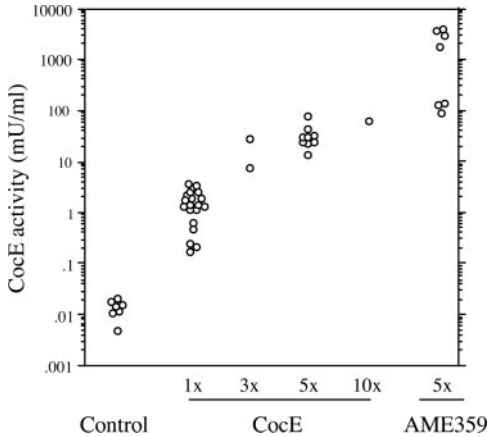

Fig. 7. Enzyme transduction versus vector dose. Rat plasma samples were collected 5 days after injection of Ad5-CMV-CocE or Ad5-CMV-AME_{359}. Vector doses are shown as multiples of the "standard", which was 2.2×10^9 PFU. Data points represent mean cocaine hydrolase activity in duplicate assays from each individual rat, graphed on a logarithmic scale. Note the million-fold range of recorded activities.

caine injection. At this time, control rats still showed a 20 mm Hg elevation of blood pressure, about 70% of the initial response, whereas pressure in the CocE and AME groups was only 13 and 5 mm Hg above baseline, respectively. The effect of AME treatment was not only larger but also progressive; in fact, blood pressure reached normal levels within a few minutes and remained so for the duration of measurement. Rats treated with the CocE vector, in contrast, failed to completely normalize pressure, which remained near 15 mm Hg for at least 10 min.

Discussion

We found that virally mediated gene transfer of cocaine hydrolases altered the metabolism and physiological effects of cocaine. The time course and pattern of this gene transduction matched those in the broader literature on adenoviral vectors, whereas the physiological effects were in line with our observations after directly administering hydrolase protein (Gao and Brimijoin, 2004). These findings support the feasibility of metabolically based gene therapies for cocaine toxicity and abuse. The data also highlight issues that must be resolved before such therapies can be realized.

Tissue distribution is one issue. CocE transduction occurred largely in the liver, consistent with the hepatotropism of type-5 adenoviral vectors (Shah et al., 2000; Connelly and Mech, 2004). The liver, specialized for metabolism of xenobiotic compounds, is a favorable site for transduced hydrolases to enhance cocaine clearance. However, expression in targets such as brain, heart, and blood vessels is also desirable. Although such tissues acquired measurable hydrolase activity when recombinant CocE was injected (Gao and Brimijoin, 2004), they were less affected by transduction. In the present study, plasma and liver of rats transduced with Ad5-CMV-CocE displayed peak cocaine hydrolase activities close to those previously seen after injection of CocE. But brain, heart, and lung showed 5-fold lower CocE levels after transduction than after enzyme injection, and diaphragm, kidney, and spleen showed 15-fold lower levels.

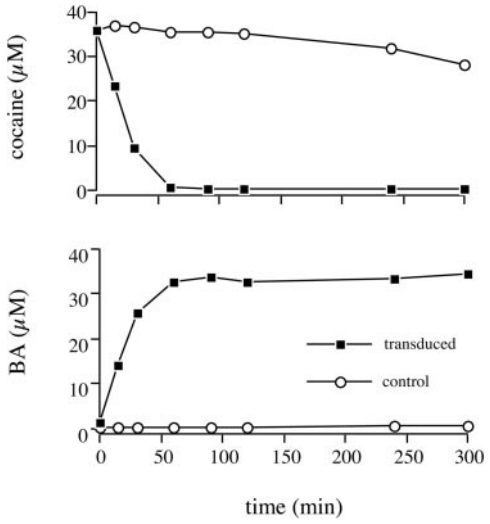

Fig. 8. Cocaine clearance from plasma in vitro. [^{3}H]Cocaine, 37 μM, was added to plasma from a rat treated 5 days earlier with 11×10^9 PFU Ad5-CMV-CocE. Levels of cocaine and the metabolite benzoic acid (BA) were determined at multiple times by radiometric assay (see *Materials and Methods*).

The different distributions of CocE activity after injections of protein or vector probably reflect differences in enzyme fate. Recombinant CocE not only has a shorter terminal half-life than transduced CocE but also exhibits a "redistribution phase" after i.v. injection. We suspect this rapid initial clearance involves adsorption onto reticuloendothelial cells in lung, liver, spleen, and kidney, and to a lesser extent elsewhere. Adsorption may be greater if the enzyme is not fully glycosylated and assembled into tetramers, or is derived from a foreign species (Kronman et al., 1995; Saxena et al., 1998). Because recombinant CocE qualifies on both counts, CocE injections probably line blood vessels with enzymatically active protein that speeds cocaine's removal, and, more importantly, hinders its local cardiovascular and neurological actions. The transduced hydrolase, on the other hand, cleared slowly, showed no redistribution phase and did not deposit in tissues. When such an enzyme is generated mainly in liver, only a robust expression can reduce the physiological impact of an i.v. drug. That could be why CocE transduction proved less effective than equivalent injections of enzyme

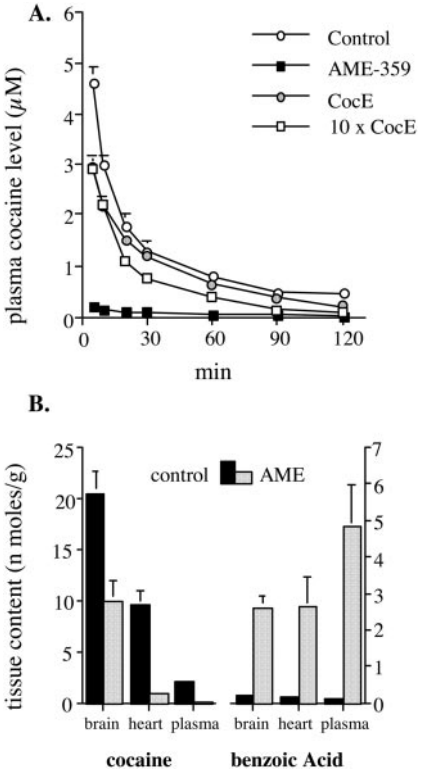

Fig. 9. Cocaine clearance in vivo. A, time course of plasma cocaine. Rats were transduced with Ad5-CMV-CocE in a dose of 11×10^9 PFU (CocE, $n = 9$) or 22×10^9 PFU (CocE high, $n = 1$). Four other rats received 11×10^9 PFU of Ad5-CMV-AME$_{359}$ (AME$_{359}$). One control rat received the same dose of empty Ad5-CMV vector; two others were untreated. Five days after transduction, a dose of 30 μCi of [^{3}H]cocaine (3.5 mg/kg) was injected i.v.; blood samples at the indicated times were assayed for radiolabeled cocaine and benzoic acid. Means ± S.E.M. are shown. The effect of CocE transduction was statistically significant at the 5-min point only (t test, $p < 0.05$), but AME$_{359}$ transduction dramatically reduced cocaine at all tested times. B, changes in tissue cocaine and benzoic acid. Three control rats and three Ad5-CMV-AME$_{359}$ rats were euthanized 5 min after injection of [^{3}H]cocaine, and heart and brain samples were collected after saline perfusion. Means ± S.E.M. are shown in units of nanomoles per gram (assuming for plasma, $\rho \approx 1$ kg/l). The reductions in cocaine content and increases in benzoic acid in transduced tissues were all significant by t test ($p < 0.005$).

protein in speeding cocaine metabolism and reducing pressor responses.

A second issue raised by our results is the time course of hydrolase transduction. Although expression was detectable for 3 weeks, the drop soon after peak expression was striking. This pattern, often seen with adenoviral vectors, is thought to reflect immunological reactions, including T cell-mediated attack on vector-containing host cells and humoral inactivation of transduced proteins (Dai et al., 1995; Yang et al., 1995). Both reactions were likely in rats given Ad5-CMV-CocE or -AME_{359}. We did not perform histology to confirm an inflammatory process, but the rats examined 5 days or more after transduction had bright yellow plasma and swollen livers. These signs point to hepatitis and are consistent with the disappearance of CocE mRNA from liver within 9 days. Humoral mechanisms remain an open question for now because we did not detect CocE-binding factors attaching to anti-rat IgG or IgM. Nonetheless CocE-reactive antibodies or lymphocytes may well explain the drop in enzyme half-life from 33 h in naive rats to less than 30 min in a previously transduced rat. Actual patients would be less likely to generate antibodies against CocE, a natural human protein with only two mutations located deep inside the catalytic gorge.

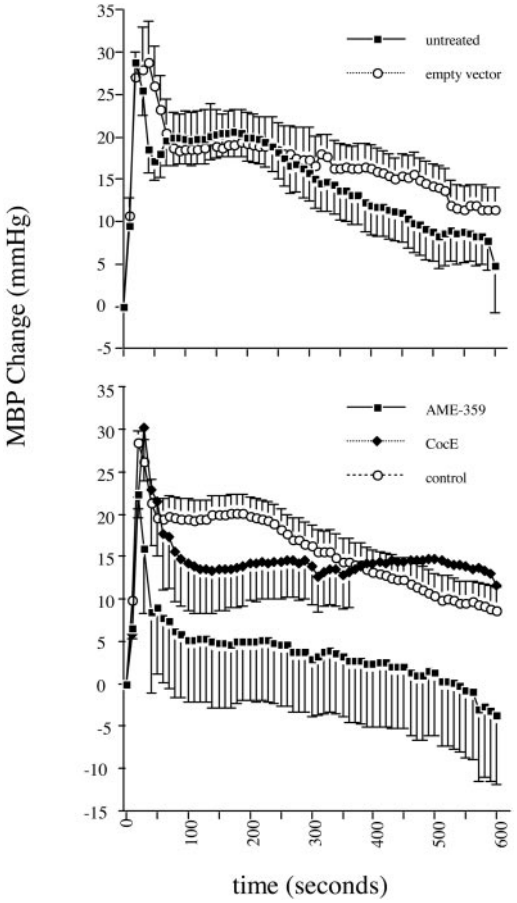

Fig. 10. Hydrolase transduction reduces cocaine's pressor effect. Rats received 11×10^9 PFU of Ad5-CMV-CocE ($n = 8$), Ad5-CMV-AME_{359} ($n = 5$), or empty Ad5-CMV vector ($n = 5$). Five days later, under urethane anesthesia, atropine (1 mg/kg) was given 10 min before cocaine challenge (3.5 mg/kg i.v.). Means ± S.E.M. of changes in blood pressure are shown at 10-s intervals. Top, comparison of the empty vector rats with 13 untreated animals, which did not differ statistically. Bottom, comparison of transduced rats with pooled controls (untreated rats and rats given empty vector). Two-way analysis of variance showed high significance ($p < 0.0001$) in the effects of transduction with Ad5-CMV-CocE (during the first 360 s after cocaine) and with Ad5-CMV-AME_{359} (throughout the observation window).

Even though the enzyme transduction was limited in duration, it was impressive in magnitude, particularly during the first week. Peak plasma levels of CocE protein were 10 times above those typical for plasma BChE in normal human subjects. The high expression and catalytic efficiency of the modified hydrolases speeded cocaine metabolism and decreased the pressor responses during drug challenge. These effects were most prominent with AME_{359}, which destroyed cocaine so rapidly it was scarcely detectable 4 min after injection.

AME_{359} owes its catalytic power to the combination of its four amino acid substitutions, which include the A328W mutation in CocE. AME_{359} has a k_{cat} of 620 min^{-1} (4 times that of CocE) and a K_m of 20 μM (equal to CocE), conferring unequalled catalytic efficiency (J. D. Pancook and S. Brimijoin, unpublished results). Multiple mutations of a mammalian protein can pose liabilities, however, by impairing transcription, translation, or proper folding. In one well studied group of drug-metabolizing enzymes, the sulfotransferases, even a single nonsynonymous nucleotide polymorphism can decrease expression markedly (Weinshilboum and Wang, 2004). Surprisingly, such factors seem not to have limited the viral transduction of the mutant hydrolase. Although AME_{359} was less well assembled into tetramers, the relative abundance of its activity in plasma suggests that this protein may have expressed even more readily than CocE.

The present results support our recent finding that injection of a cocaine hydrolase will reduce cardiovascular responses to cocaine in a highly specific manner, by accelerating metabolic elimination (Gao and Brimijoin, 2004). The results also show that similar but more lasting effects can be achieved by viral transduction. Such effects could aid in reducing cocaine toxicity or in rescuing abusers from an overdose. When it comes to addiction itself the question remains open. Can gene transfer of a cocaine hydrolase reduce behavioral responses to cocaine, and will it lower the risk of addiction or relapse? The objective seems both desirable and feasible in principle, but many hurdles must be overcome to achieve it.

A gene therapy for cocaine addiction must be safe, effective, and sustainable. To date, no systemic gene therapy for any condition can be called "safe". Hence, applications of this technology for all but life-threatening conditions must wait until genes can be delivered without substantial risk. Furthermore, the prospects for reducing the psychic rewards of cocaine abuse by increased metabolism are uncertain, despite the impressive decrease in cardiovascular responses in rats with high hydrolase activity. One reason is that the brain is a preferred site of cocaine uptake. Thus, the brains of transduced rats with drastically reduced pressor responses, and correspondingly little drug in the heart, still accumulated 45% as much cocaine as did controls. Much of this residual load may be functionally irrelevant (e.g., dissolved in myelin), but some is likely to reach dopamine transporters and other targets. Therefore, to create a still more effective barrier to the central nervous system effects of cocaine, hydrolase activity may be needed in the brain vasculature or the brain parenchyma itself. Expression in the nervous system will be essential if tests of self-administration in animals

indicate that transduction solely in the periphery intensifies drug-seeking by eliminating side effects while sparing rewards. Potential solutions to this problem are adeno-associated viral vectors (Grimm and Kay, 2003) or lentiviral vectors (Jakobsson et al., 2003), both of which can transduce nondividing cells and generate long-term expression in the nervous system (Thomas et al., 2003).

A metabolic gene therapy might help motivated individuals in voluntary drug cessation programs by shifting dose-response curves to the right, reducing psychic benefits, and, perhaps eventually, extinguishing the behavior of drug intake. Loopholes would remain, unfortunately. Even a "perfect" hydrolase could be overwhelmed by large enough doses of drug or by accompanying inhibitors. But the extra cost and inconvenience of such measures could be expected to limit their appeal and reduce the incentive for relapse. In any case, an investigation of these issues should shed new light on the pharmacokinetic and pharmacodynamic factors that make cocaine so dangerous and problematic.

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Address correspondence to: Dr. S. Brimijoin, Department of Molecular Pharmacology, Mayo Clinic, 200 First St. S.W., Rochester MN 55905. E-mail: brimijoi@mayo.edu