Cocaine Hydrolase Gene Transfer Demonstrates Cardiac Safety and Efficacy against Cocaine-Induced QT Prolongation in Mice

Vishakantha Murthy, Santiago Reyes, Liyi Geng, Yang Gao, and Stephen Brimijoin

Department of Molecular Pharmacology and Experimental Therapeutics, Robert and Arlene Kogod Center on Aging (V.M., L.G., Y.G., S.B.), and Marriott Heart Disease Research Program, Division of Cardiovascular Diseases (S.R.), Mayo Clinic, Rochester, Minnesota

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ABSTRACT

Cocaine addiction is associated with devastating medical consequences, including cardiotoxicity and risk-conferring prolongation of the QT interval. Viral gene transfer of cocaine hydrolase engineered from butyrylcholinesterase offers therapeutic promise for treatment-seeking drug users. Although previous preclinical studies have demonstrated benefits of this strategy without signs of toxicity, the specific cardiac safety and efficacy of engineered butyrylcholinesterase viral delivery remains unknown. Here, telemetric recording of

electrocardiograms from awake, unrestrained mice receiving a course of moderately large cocaine doses (30 mg/kg, twice daily for 3 weeks) revealed protection against a 2-fold prolongation of the QT interval conferred by pretreatment with cocaine hydrolase vector. By itself, this prophylactic treatment did not affect QT interval duration or cardiac structure, demonstrating that viral delivery of cocaine hydrolase has no intrinsic cardiac toxicity and, on the contrary, actively protects against cocaine-induced QT prolongation.

Introduction

Among drugs of abuse, cocaine stands out for its cardiovascular toxicity, often leading to emergency department visits (Hahn and Hoffman, 2001). A common effect of cocaine abuse is prolongation of the QT interval in the electrocardiogram (ECG), which, when extreme, leads to *torsades de pointes*, a life-threatening ventricular dysrhythmia (Kloner et al., 1992; Bauman and DiDomenico, 2002; Phillips et al., 2009). There is currently no available pharmacotherapy that would prevent these adverse outcomes (Mendelson and Mello, 1996; Wood et al., 2009).

To fill this gap, we have pursued gene therapy means to enhance cocaine metabolism in an effort to block both the reward and the toxicity of this drug. Animal studies show some benefit from butyrylcholinesterase (BChE), a common plasma enzyme. Native BChE destroys cocaine slowly, but computer simulations and site-directed mutagenesis have uncovered critical active-site mutations that collectively enhance cocaine hydrolysis by approximately 1500-fold (Xie et al., 1999; Sun et al., 2002; Yang et al., 2010; Zheng et al., 2010). Such a cocaine hydrolase (CocH) cleaves cocaine into ecgonine methyl ester (EME) and benzoic acid, both of which lack reward value and toxicity (Murthy et al., 2015). This enzyme is now a central focus in efforts to develop a gene transfer therapy for cocaine abuse.

The preferred intervention strategy requires long-term CocH expression. Instead of multiple injections or slow-release preparations, gene transfer by adeno-associated viral vector (AAV) or helper-dependent adenoviral vector looks more promising. Over a range of species, this technology can drive CocH to extremely high levels, for years after a single treatment and without apparent toxicity (Murthy et al., 2014a,b). The result is a greatly accelerated cocaine hydrolysis and a near-complete suppression of physiologic, behavioral, and toxicological responses to cocaine (Gao and Brimijoin, 2004; Carroll et al., 2012; Brimijoin et al., 2013; Gao et al., 2013; Geng et al., 2013). We consider that a successful translation of this therapy to treatment-seeking cocaine users might aid them in becoming abstinent and, later, reduce their risk of relapse into drug taking.

Translating CocH gene transfer technology into clinical application demands careful evaluation in animal models. Multiple studies have shown negligible physiologic, biochemical, neurobehavioral, and neuromuscular toxicity from BChE injections and gene transfer (Saxena et al., 2005, 2011; Brimijoin et al., 2008; Weber et al., 2011; Murthy et al., 2014a,b, 2015). Nonetheless, the issue of cardiovascular safety needs closer attention. This study used telemetry to capture ECGs from conscious free-moving mice undergoing long-term cocaine treatment. There were two objectives. The first was to determine whether CocH-based gene therapy is safe to the heart's structure and function. The second was to test our hypothesis that, beyond not being harmful, CocH gene transfer protects against cocaine cardiotoxicity exemplified by prolongation of the QT interval on the ECG.

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ABBREVIATIONS: AAV, adeno-associated viral vector; BChE, butyrylcholinesterase; CocH, cocaine hydrolase; ECG, electrocardiogram; EME, ecgonine methyl ester; ERG, Ether-à-go-go-Related Gene; QTc, QT interval corrected for heart rate.

Materials and Methods

Animals and Housing. Balb/c male mice (aged 7 to 8 weeks, weighing 26.2 ± 0.5 g) purchased from Harlan Laboratories (Madison, WI) were group-housed in plastic cages with access to water and food (Purina Laboratory Chow; Purina Mills, Minneapolis, MN) in rooms controlled for temperature (24°C), humidity (40%–50%), and illumination (lights on 6 AM, lights off 6 PM). To monitor food and water consumption, mice were housed individually from 3 days before surgery until the end of the experiment. The protocol (A20812) was approved by the Mayo Clinic Institutional Animal Care and Use Committee, and experiments were conducted in accordance with the Principles of Laboratory Animal Care in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility.

Surgery and Telemetry. To monitor heart rate and ECG results continuously in conscious mice, ETA-F10 telemetry devices (weight, 1.6 g; volume, 1.1 ml; Data Sciences International, St. Paul, MN) were implanted in the peritoneal cavity, and wire leads were tunneled subcutaneously in a “lead II configuration” (O’Cochlain et al., 2004; Kane et al., 2006; Alekseev et al., 2010). Implantation surgery was conducted strictly according to Association for Assessment and Accreditation of Laboratory Animal Care International–approved aseptic conditions, 1 day after removal of anterior neck and abdominal hair. Anesthesia was achieved with isoflurane (5% for induction, 1.5% for maintenance in pure oxygen) and body temperature was maintained with a water-jacketed table at 37°C . Implantation surgery was highly successful. Of the 27 mice subjected to telemetry device implantation, only 1 mouse failed to recover fully and was euthanized after 3 days. Two other mice were euthanized because of persistent chewing on the telemetry leads. None died as a result of viral transduction or cocaine administration. All 24 of these telemetry implanted mice were randomly assigned to three experimental groups. In general, core body temperature stabilized at approximately 38°C by 4 to 5 weeks. At this point, the vector group received dexamethasone (10 mg/kg and 5 mg/kg, i.p.) 16 hours and 2 hours before virus injection and a further 5 mg/kg 24 hours later. These treatments suppressed acute immune responses that impaired enzyme transduction. Telemetry data were acquired weekly for 3 weeks at 2 kHz after 4 to 5 weeks of recovery from telemetry implantation (when core temperature stabilized) and 4 weeks after vector injection, as shown in Fig. 1.

Viral Gene Transfer. AAV-8 vector incorporating cDNA for mouse BChE with amino acid substitutions for improved cocaine hydrolysis (A199S/S227A/S287G/A328W/Y332G) was prepared as previously described (Geng et al., 2013). This CocH sequence was ligated into pAAV-VIP vector (Balazs et al., 2012) between NotI and BamHI restriction sites. An AAV-VIP-CocH mutant plasmid was cotransfected into human embryonic kidney 293 T cells with pHelper and pAAV2/8, using X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN). AAV virus was purified from cell lysates by ultracentrifugation against Optiprep Density Gradient Medium-Iodixanol (Sigma-Aldrich, St. Louis, MO). After recovery from telemetry implantation, the 12-week-old mice identified for gene transfer treatment were given vector (200 μl , 1013 viral particles) via the tail vein.

Blood Collection. Blood (<0.1 ml) was taken from the cheek with a 21-gauge mouse lancet and bleeding was stopped with sterile gauze. Samples were centrifuged for 15 minutes ($8000 \times g$) and plasma was stored at -20°C . CocH activity was assayed in a solvent-partitioning assay with ^3H -cocaine (50 nCi) and cold cocaine (18 μM) as described (Brimijoin et al., 2002). A related procedure determined levels of ^3H -cocaine and benzoic acid, with diisopropyl fluorophosphate added to halt enzymatic breakdown.

Histology. One day after the last cocaine administration, whole hearts were removed, rinsed, blotted dry, and flash-frozen in liquid nitrogen–chilled isopentane. Micrographs of 5- μm transverse sections of the heart, including left and right ventricles, stained with

hematoxylin and eosin were taken with a KS-4000 Axiocam digital camera on an Axioplan 2 light microscope (Carl Zeiss MicroImaging, Thornwood, NY). Quantitative histologic measurements were made under treatment-blind conditions.

Drugs. Cocaine HCl was obtained from the National Institute on Drug Abuse Drug Supply Program (Research Triangle Park, NC).

Intraperitoneal Injections. For all experiments, the intended dose of cocaine was freshly dissolved in 0.9% NaCl before injection. Concentrations were adjusted to deliver approximately 300 μl per subject for a 30-mg/kg cocaine dose. Control mice received comparable volumes of saline.

Data Analysis. Electrocardiographic analysis was performed at three different time points for each recording session (approximate time points: initial, 3–7 minutes; middle, 12–17 minutes; and final, 22–27 minutes) under normal sinus rhythm. The RR interval refers to the distance between adjacent R waves. The QT interval was defined as the time from the start of the Q wave to the end of the T wave (return to isoelectric point) on the electrogram. The QT interval was corrected for heart rate (QTc) by using the following formula (Mitchell et al., 1998; Kane et al., 2006):

$$\text{QTc}(\text{ms}) = \frac{\text{QT}(\text{ms})}{\sqrt{\text{RR}(\text{ms})/100}}$$

Statistical Analysis. Data were analyzed using *t* tests with Microsoft Excel software (Redmond, WA) and two-way analysis of variance with Holm–Sidak testing for post hoc analyses with GraphPad Prism software (version 6.0; GraphPad Inc., La Jolla, CA). Statistical significance was accepted if $P < 0.05$.

Results

Experimental Sequence. After acclimation on arrival, mice received telemetry implants. Upon recovery, mice were randomly assigned to the three groups: vector, saline, and cocaine ($n = 8$ each). Mice in the vector group received CocH gene transfer; when stably high enzyme expression was achieved, mice were entered into the 3-week course of cocaine treatment (flowchart, Fig. 1). The other 16 mice were divided into pure negative controls (saline only) and positive controls (single saline injection in place of vector, followed by the course of cocaine).

Time Course of CocH Expression. Initial experiments addressed the levels and duration of CocH in plasma after gene transfer. Plasma cocaine hydrolytic activity reached an average level of 35 U/ml, 2–4 weeks after administration of AAV-CocH vector, and remained stable for the remainder of the experiment (data not shown). Consistent with our recent reports (Geng et al., 2013; Murthy et al., 2014a) the average rise in enzyme-driven cocaine hydrolysis capacity was approximately 100,000-fold above the pretreatment level generated by endogenous BChE (0.0004 U/ml).

Temperature. Shortly after telemetry implantation, mice developed a moderate fever (approximately 40°C) but returned to approximately 38°C within 5 weeks. After confirming stable enzyme expression but before cocaine administration, telemetry showed an average body temperature of $37.9 \pm 0.2^{\circ}\text{C}$, with no difference between vector-treated and control animals. Weekly recordings throughout the experiment continued to reveal no significant differences between treatment groups (vector versus control) or within groups (before and after cocaine). Thus, neither vector treatment nor cocaine injection had discernible effects on body temperature (Table 1).

Fig. 1. Experimental flowchart. Mice were acclimated on arrival, followed by implantation of telemetry devices. After surgical recovery, mice were randomly assigned to vector (AAV-CocH, 10^{13} viral particles, i.v., $n = 8$) or saline pretreatment ($n = 16$). Four weeks later, plasma CocH expression was confirmed in the vector group and baseline ECG was recorded in all groups. As indicated, saline controls received only saline intraperitoneally, twice daily (approximately 300 μ l per injection). Cocaine and vector groups ($n = 8$ each) both received 30 mg/kg cocaine intraperitoneally, twice daily. All mice were recorded each week (30-minute sessions).

Heart Rate. As with body temperature, neither viral vector nor cocaine administration induced a change in heart rate. At baseline and during the cocaine treatment regimen, no significant fluctuations were observed in cocaine-positive controls (500 ± 35 bpm at baseline, 540 ± 33 bpm at day 7, 549 ± 30 bpm at day 14, and 513 ± 36 bpm at day 21), saline controls (499 ± 46 bpm at baseline, 482 ± 47 bpm at day 7, 522.36 ± 40.32 bpm at day 14, and 543.21 ± 22 bpm at day 21), or vector-treated mice (560 ± 20 bpm at baseline, 556 ± 13 bpm at day 7, 529 ± 426 bpm at day 14, and 530 ± 28 bpm at day 21) (Fig. 2). Likewise, heart rate in vector-treated mice did not change from pretreatment levels.

Cocaine-Induced Prolongation of QTc Interval. Mice given only saline showed stable QTc intervals throughout the 3 weeks of injections and observation (Fig. 3). By contrast, mice treated with cocaine alone developed a statistically significant QTc prolongation that increased progressively over the course of drug administration: 30.2 ± 2.6 milliseconds at baseline, 37.5 ± 3.3 milliseconds at day 7 ($P = 0.01$), 35.0 ± 3.5 milliseconds at day 14 ($P = 0.44$), and 47.4 ± 3.3 milliseconds at day 21 ($P = 0.02$). Vector alone did not affect QT intervals: 22.6 ± 0.9 milliseconds at baseline and 27.8 ± 3.4 milliseconds at day 7 after virus delivery ($P = 0.09$). Most importantly, after 3 weeks of cocaine treatment, this group of mice showed no QT prolongation with a final value of 26.2 ± 1.6 milliseconds, which was nonsignificantly lower than the precocaine level.

In addition, QRS analysis revealed no cocaine-induced changes in QRS duration either in unprotected mice (12.8 ± 1.0 milliseconds at baseline versus 12.4 ± 1.0 milliseconds at 3 weeks; $P = 0.71$), or in mice pretreated with CocH vector (11.2 ± 0.3 milliseconds at baseline versus 13.0 ± 1.4 milliseconds at 3 weeks; $P = 0.29$).

Histology. Fresh-frozen cryostat sections stained with hematoxylin and eosin revealed no indication of cardiac

myopathy in the left ventricles of cocaine-positive controls, saline controls, or vector-treated mice (Fig. 4, A, B, and D). Furthermore, the cardiomyocyte cross-sectional area was not affected by administration of cocaine alone or in the presence of vector compared with saline (Fig. 4C; $P = 0.70$, analysis of variance). This outcome confirmed expectations that 3 weeks of cocaine dosing should be adequate to induce electrophysiological abnormalities but not grossly evident cardiomyopathy.

Discussion

To pave the way for an eventual human trial of CocH gene transfer therapy, we have conducted several preclinical studies to examine neurobehavioral, neuromuscular, metabolic, and physiologic parameters (Murthy et al., 2014a,b, 2015). Despite the high levels of CocH achieved in mice, rats, and rhesus monkeys, no indications of toxicity or physiologic dysfunction emerged, but cardiac effects remained to be tested. Because cocaine is cardiotoxic, it is important to eliminate the possibility that hydrolase gene transfer might disturb heart rhythms. A key metric of treatment-induced electrophysiological dysfunction is prolongation of the QT interval. Drug-induced QT prolongation can lead to fatal *torsades de pointes*, a primary cause of failure in drug discovery projects. Because the U.S. Food and Drug Administration routinely mandates QT reports as a condition for an investigational new drug permit, it is important to document the effects of CocH and cocaine, alone or combined, on QT intervals in mice.

Cocaine acts to block presynaptic reuptake of dopamine, norepinephrine, and serotonin in the autonomic and central nervous systems and enhances adrenergic tone. Excessive sympathetic stimulation can lead to life-threatening cardiac stress. Thus, an agent that limits cocaine's actions at adrenergic synapses should have clinical value. Our proposed gene therapy focuses on reducing behavioral impact by destroying cocaine before it penetrates the blood-brain barrier. This strategy is highly effective in rodent models, in which one treatment with vector-generated CocH can accelerate cocaine hydrolysis by 1000-fold or more and suppress lever pressing for cocaine reward for years (Zlebnik et al., 2014). However, until now, it had not been determined whether such treatment would be cardioprotective or, alternatively, might enhance risk in users taking larger cocaine doses to compensate for their diminished reward value.

Here, we addressed two fundamental questions: 1) Do high circulating levels of cholinesterase after viral gene transfer have any adverse cardiovascular effects? 2) Can gene transfer

TABLE 1

Body temperature in vector mice ($n = 8$) and age-matched mice treated with saline or cocaine ($n = 7$ each)

Data represent means $\pm$ S.E.M.

Treatment	Temperature			
	Baseline	Week 1	Week 2	Week 3
	$^{\circ}\text{C}$			
Saline ($n = 7$)	37.7 ± 0.4	37.8 ± 0.2	37.8 ± 0.2	38.0 ± 0.2
Cocaine ($n = 7$)	37.8 ± 0.4	38.0 ± 0.4	38.1 ± 0.3	37.6 ± 0.4
Vector ($n = 8$)	38.2 ± 0.4	37.7 ± 0.2	38.0 ± 0.3	37.8 ± 0.2

Temperature data were averaged across 30-minute intervals. At baseline, mean temperature across all groups was $37.9 \pm 0.2^{\circ}\text{C}$. No group differences were observed ($P = \text{N.S.}$ for all possible comparisons by two-way analysis of variance).

Fig. 2. Heart rate in vector mice ($n = 8$) and age-matched saline and cocaine controls ($n = 7$ each). Data are from 30 minutes of recording (mean $\pm$ S.E.M.). Baseline heart rates ranged from 450 to 600 bpm. No significant differences were detected ($P = \text{N.S.}$ for all possible comparisons by two-way analysis of variance).

of CocH alleviate the consequences of continued cocaine use, specifically the risk-conferring prolongation of QT intervals?

As a partial approach to the first question, we examined the effect of vector and cocaine treatment on cardiac structure. Chronic cocaine abuse is known to induce cardiac hypertrophy in humans. In a mouse line especially susceptible to cardiac stressors, Reyes et al. (2009) recently found that a 3-week regimen of sublethal cocaine doses induced left ventricular damage and impaired ejection velocity in mice lacking

functional ATP-sensitive potassium channels, which are established molecular sensors that protect the heart against conditions that impose an energetic overload. Our findings are in line with the control group in that prior study, which exhibited no cardiac histopathology at the administered cocaine dose. This was true in unprotected positive controls and also in the vector-treated mice exposed to a moderately high level of cocaine. Of particular importance for translational prospects, our results provide evidence that vector treatment and expression of the BChE transgene designated as CocH do not induce cardiac histopathology by themselves or enhance the inherent cardiotoxicity of cocaine. This outcome supports our view that the viral delivery of CocH is not likely to impose a substantial risk of cardiotoxicity in human users, even if they continue taking cocaine after treatment. In fact, the treatment might well provide a degree of protection.

Clinical studies clearly document QT prolongation in cocaine users (Erwin and Deliargyris 2002; Taylor et al., 2004; Magnano et al., 2006; Levin et al., 2008), but murine models of cocaine-induced QT interval prolongation have not been reported. Here, addressing question two, weekly ECG recordings during the course of cocaine treatment showed a cumulative, dose-dependent prolongation of QT, but no change in heart rate, in mice that did not receive CocH vector. That outcome is consistent with another report that 10 days of 40-mg/kg cocaine dosing caused no heart rate fluctuations (Sutliff et al., 2003). Interestingly, that treatment failed to induce QT prolongation. Furthermore, vector pretreatment to secure the presence of CocH in the circulation before exposure to cocaine prevented the drug-induced prolongation of the QT interval. Interestingly, QRS duration was not affected. This outcome suggests that the QT interval prolongation as a result of

Fig. 3. (A) Representative ECGs from each treatment group: saline controls, positive controls given cocaine, and vector-expressing mice given cocaine. The horizontal scale bar represents 50 milliseconds, and the vertical scale bar represents 0.1 mV. (B) Description of ECG component waves, highlighting P, Q, R, S, and T waves, as well as QRS and QT intervals. (C) QT intervals measured manually and corrected for heart rate (QTc; see the *Materials and Methods*) from vector mice ($n = 7-8$), saline controls ($n = 5-7$), and cocaine-treated mice ($n = 4-8$) over the course of cocaine treatment. Data represent means $\pm$ S.E.M. * $P < 0.05$, versus same group baseline.

Fig. 4. (A–C) Representative micrographs of the left ventricular posterior free wall in experimental groups as labeled (saline, $n = 4$; cocaine, $n = 3$; vector, $n = 3$, respectively). (D) Cross-sectional area is not significantly altered after repeated exposure to cocaine alone or in the presence of vector. Bar, 200 μm . Original magnification, $\times 40$.

cocaine administration was related to an increase in repolarization time, which is in line with previous reports (Guo et al., 2006; Haigney et al., 2006). It is possible that the same mechanism is responsible for the effects seen in our study, because mice have been shown to also express Ether-à-go-go-Related Gene (ERG, or Kcnh2)-encoded Kv11.1 voltage-gated potassium channels. This implies that gene transfer of CocH should indeed alleviate the adverse cardiovascular action of cocaine, adding further support for future clinical applications of this gene-based therapy.

In vector-treated mice, cocaine degrades rapidly into EME and benzoic acid. Some older literature has suggested that EME does affect blood vessel smooth muscle (Zakusov et al., 1978; Kurth et al., 1993), but we recently found no transient blood pressure changes after cocaine doses up to 80 mg/kg i.v. in mice pretreated with CocH vector (Murthy et al., 2015). Because cocaine disappeared from the blood stream within seconds of administration, the equimolar mixture of cocaine metabolites generated by CocH action must be neither hypertensive nor hypotensive. Our present experiments, delivering 60 mg cocaine per kg per day, generated approximately 40 mg/kg EME for 3 weeks without affecting body temperature, heart rate, or QT intervals. This result is strong evidence that rapid conversion of large cocaine doses into EME and benzoic acid is unlikely to pose significant risk for vector-treated human subjects.

Clinically, chronic cocaine users exhibit thermoregulatory aberrations, impaired sweating, and cutaneous vasodilation (Crandall et al., 2002). However, in our murine model of drug

use, there were no sustained fluctuations in temperature throughout the cocaine delivery regimen, either in the positive controls or in the vector-treated mice. It is also worth noting that given the autonomic nervous system's involvement in thermal stress and thermoregulation, vector treatment and excess of transgene BChE had no long-term effect on body temperature.

We conclude that exposure to viral vector, elevated expression of modified BChE, and cocaine metabolites enzymatically generated during a prolonged course of drug injections do not have detectable adverse effects on the cardiovascular system. On the contrary, CocH vector treatment in this murine model effectively prevented cocaine-induced QTc prolongation. This outcome is encouraging in regard to an eventual translation of CocH vector into human use. However, because significant differences in cardiac electrophysiology between mice and humans are well known, further testing in larger animal models is warranted before human trials should be considered. Nonetheless, the safety and efficacy documented here provide encouragement for the idea that CocH gene transfer might someday form the basis of a robust treatment to aid cocaine users to achieve relapse-free abstinence from this destructive drug.

Authorship Contributions

Participated in research design: Murthy, Reyes, Brimijoin.

Conducted experiments: Murthy, Reyes, Geng, Gao.

Performed data analysis: Murthy, Reyes.

Wrote or contributed to the writing of the manuscript: Murthy, Reyes, Brimijoin.

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Address correspondence to: Dr. Vishakantha Murthy, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail: murthy.vishakantha@mayo.edu