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Effects of anti-cocaine vaccine and viral gene transfer of cocaine hydrolase in mice on cocaine toxicity including motor strength and liver damage

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ABSTRACT

In developing an vivo drug-interception therapy to treat cocaine abuse and hinder relapse into drug seeking provoked by re-encounter with cocaine, two promising agents are: (1) a cocaine hydrolase enzyme (CocH) derived from human butyrylcholinesterase and delivered by gene transfer; (2) an anti-cocaine antibody elicited by vaccination. Recent behavioral experiments showed that antibody and enzyme work in a complementary fashion to reduce cocaine-stimulated locomotor activity in rats and mice. Our present goal was to test protection against liver damage and muscle weakness in mice challenged with massive doses of cocaine at or near the LD50 level (100–120 mg/kg, i.p.). We found that, when the interceptor proteins were combined at doses that were only modestly protective in isolation (enzyme, 1 mg/kg; antibody, 8 mg/kg), they provided complete protection of liver tissue and motor function. When the enzyme levels were ~400-fold higher, after in vivo transduction by adeno-associated viral vector, similar protection was observed from CocH alone.

1. Introduction

In vivo drug-interception by antibodies or enzymatic destruction is emerging as a potential treatment for substance abuse, with the concept of preventing addiction relapse in recovering users who re-encounter their particular drug of choice [1,2]. Cocaine abuse is a promising target because cocaine is subject to one-step enzymatic inactivation, and because a cocaine vaccine has already shown some success in a clinical trial [3]. We are investigating a cholinesterase-derived cocaine hydrolase (CocH) for possible synergy with anti-cocaine antibodies, since both agents reduce the drug's access to brain. Enzymes destroy limitless quantities in time, while antibodies bind rapidly but can be saturated by large or repeated drug doses. These complementary properties led to the idea that combined treatments would be particularly efficient [4]. In theory, when both agents are present, antibody can sequester part of a drug bolus while enzyme hydrolyzes free molecules. As the equilibrium shifts, drug will off-load from the antibody to be destroyed in turn, restoring the original state. Thus, these agents might act synergistically to shield the brain (reducing addiction liability) and also protect peripheral tissues, such as liver, that are direct targets of cocaine toxicity [5–7]. We recently presented supportive behavioral evidence for this idea [8]. The current study was designed to extend those observations by determining whether anti-cocaine antibody and cocaine hydrolase would also cooperate to reduce the toxic effects of cocaine in mice, with particular respect to muscle impairment and liver damage. Here we present key findings from initial experiments on the potential for additive or synergistic therapeutic effects from CocH and anti-cocaine antibodies.

2. Materials and methods

2.1. Drug source

Cocaine HCl was obtained from NIDA (National Institute on Drug Abuse, Bethesda MD). Purified CocH, a quadruple mutant of human butyrylcholinesterase (A199S/S287G/A328W/Y332G) first reported by Pan et al. [9] and characterized further by Yang et al. [10], was obtained in the form of a C-terminal fusion with human serum albumin (D. LaFleur, Cogenesys Inc.) from clonal lines of stably transfected Chinese hamster ovary cells. The enzyme was purified on DEAE Sepharose followed by ion exchange chromatography as previously described [11] and stored at −80 °C until used.

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2.2. Animals

Balb/c male mice obtained at 6–7 weeks of age from Harlan Sprague Dawley (Madison WI) were housed in plastic cages with free access to water and food (Purina Laboratory Chow, Purina Mills, Minneapolis, MN, USA) in rooms controlled for temperature (24 °C), humidity (40–50%), and light (light/dark, 12/12-h with lights on at 6:00 a.m.). The animal use protocol (A4309) was approved by the Mayo Clinic Institutional Care and Use Committees. All experiments were conducted in accordance with the Principles of Laboratory Animal Care in laboratories accredited by the American Association for the Accreditation of Laboratory Animal Care.

2.3. Antibody and vaccine

Anti-cocaine antibodies with sub-micromolar affinity and 8100-1 KLH SNC vaccine (norcocaine hapten-conjugated keyhole limpet hemocyanin) were prepared at Baylor College of Medicine as previously described [12]. The vaccine was injected at a volume of 80 μl into the upper thigh of each hind leg, in a total dose of 100 μg/mouse. After three weeks the same dose was given as a booster immunization. To determine levels of specific anti-cocaine antibodies, a ~50 μl blood sample was taken from each mouse, plasma was obtained after centrifugation, and diisopropylfluorophosphate (10^{-5} M) was added to inactivate cocaine hydrolysis. Samples were then incubated 50 min with ^{3}H-cocaine in near saturating concentration (5 μM), 50 μl aliquots were centrifuged on a Centricon Sepharose spin-column at 1000*g* for 4 min, and 30 μl of the void volume fraction was mixed for scintillation counting in 4 ml "BioSafe" fluor (RPI Inc., Mt Prospect IL). Validation experiments showed that >80% of the sample protein (including IgG with bound cocaine) passed into the collection tube, while >98% of free cocaine remained on the column. Assay signals (counts per min) were linearly proportional to IgG concentration over a wide range and were calibrated with reference to a known quantity of purified anti-cocaine IgG run alongside the test samples.

2.4. Drug and protein injections

Cocaine HCl was delivered i.p. (100 mg/kg or 2 doses of 60 mg/kg spaced 10 min apart). Protein pretreatments, 2 h prior to cocaine, were: (a) CocH, 1 mg/kg or (b) cocaine antiserum equivalent to 8 or 16 mg/kg anti-cocaine IgG. Delivery was initially i.v. but, where indicated (see Results) later experiments used i.p. injection as it was less stressful and yielded similar plasma levels at the time of cocaine injection. Blood samples (<100 μl) were collected at appropriate times from the tail vein. Plasma was separated by centrifugation for 10 min at 8000*g* and then immediately used for enzyme determinations or stored at −20 °C pending analysis.

2.5. Viral gene transfer

Gene transfer utilized an adeno-associated viral vector, pAAVio-CASI-CocH C-W-SV40 provided by Drs. Alejandro B Balazs and David Baltimore, Cal. Tech, Pasadena CA). This vector was modified to incorporate cDNA for C-terminally truncated CocH (enzyme sequence only, lacking the final 52 residues). The AAV8 virus was then prepared by co-transfecting this vector along with pHelper and pAAV2/8 vectors, provided by Dr. Stephen J. Russell at Mayo Clinic, into HEK293T cells. Viral genome copy numbers were determined by quantitative polymerase chain reaction (QPCR) assays using a forward primer targeting upstream viral DNA and a reverse primer targeting the N-terminal region of the CocH (human butyrylcholinesterase) sequence. Vector delivery was accomplished by rapid injection of 10^{13} viral genome copies through the tail vein in an initial volume of 0.1 ml followed by 0.1 ml of 0.9% sterile NaCl solution.

2.6. Grip strength and functional observations

After cocaine challenge locomotor activity and gait were assessed with a functional observational battery, and muscle strength was measured with a strain gage (model GS3, BIOSEB, Vitrolles Cedex, France). Body weight, determined before testing because of possible effects on the outcome, did not differ among treatment groups.

For strength determinations mice were held by the tail above the wire grid of the apparatus, gently lowered until all four legs grasped the grid, and then pulled away along the horizontal axis. Maximal achieved force was displayed and recorded. The procedure was repeated three times on each test day, and mean peak force was used for statistical analysis.

2.7. Liver function and pathology

To assess liver status, plasma alanine transaminase activity (ALT) was measured by the method of Bergmeyer et al. [13] using a kit from Thermo Scientific (Middletown, VA) with positive and negative controls. For postmortem pathology mice were euthanized with sodium pentobarbital 200 mg/kg ("Sleepaway", Fort Dodge Animal Health, IA) followed by intracardial perfusion with 50 ml isotonic NaCl. Livers were then harvested and stored at −80 °C. Frozen sections (14 μm) were later cut on a Leica cryostat and stained with hematoxylin and eosin (H&E) according to standard procedures [14]. Areas of lobular necrosis were traced and analyzed with the NIH Image-J image-processing program by an observer blinded to treatment conditions.

2.8. Statistics

Statistical significance was evaluated by ANOVA with Fisher's PLSD as a post hoc test.

3. Results and Discussion

3.1. Protection of muscular strength and function

Low doses of cocaine provide at least subjective enhancement of motor performance [15], but high doses impair it and are directly toxic to skeletal muscle [16]. We examined whether treatment with CocH or anti-cocaine vaccine would preserve hind limb grip strength in mice given high-dose cocaine (100 mg/kg, i.p.). Unprotected mice were not tested because this cocaine dose was near the LD50 [17]. Either enzyme or vaccine treatment allowed all mice to survive the cocaine challenge, but at the doses delivered neither by itself was able to prevent reduced grip strength (Fig 1). Combined treatment in identical amounts, however, fully protected muscle function, at least by this measure, and also prevented tremors and other outward signs of dysfunction seen in singly treated mice. These encouraging results warrant further studies with other muscle biomarkers and a histological analysis in order to confirm our tentative conclusion that muscle toxicity was completely abrogated.

3.2. Protection against cocaine induced liver damage

As cocaine is hepatotoxic, especially in mice [18,19], it was important to determine whether gene transfer with hepatotropic viral vectors would enhance or reduce liver toxicity. As a biochemical marker of liver damage we measured plasma levels of the