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Induction of Acetylcholinesterase Activity and Apoptosis in Carboxylesterase and Butyrylcholinesterase Knockout Mice Treated with Cocaine

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Abstract

Background: Cocaine is a commonly used illegal recreational drug and its consumption can produce various adverse health effects in animal and clinical studies. To date no information is available on whether exposed to cocaine will result in abnormally high plasma AChE activity in animals and whether it is characteristic of apoptosis. Our goals were to examine the relationship between enhanced AChE activity and cocaine-induced apoptosis and the possible underlying mechanisms.

Methods: For this purpose, carboxylesterase and butyrylcholinesterase deficient ES1-/-BChE-/- mice in strain C57BL/6 were treated intraperitoneally with 25 mg/kg cocaine daily for 8 days and sacrificed on day 9. Plasma AChE activity and body temperature were measured before and after treatment. Tissue sections from brain, heart, kidney, and liver were stained for AChE activity and apoptosis.

Results: Mice had a 1°C decrease in surface body temperature at 10 min after cocaine treatment and the temperature returned to base line by 30 min. Plasma AChE activity in mice increased about 1.5-fold on days 7-8 and 1.75-fold on days 9 after cocaine treatment. More apoptotic cells were observed in liver sections of treated mice compared to controls. TUNEL-positive cells in the liver also stained heavily for AChE activity.

Conclusions: AChE activity and apoptosis were both induced in carboxylesterase and butyrylcholinesterase knockout mice treated with cocaine. Their relationship might provide some novel information of cocaine-associated toxicity. Abnormally high plasma AChE activity may be an effect biomarker of cocaine exposure.

Keywords: Acetylcholinesterase induction; Apoptosis; Cocaine; Carboxylesterase knockout mice; Hepatotoxicity

Abbreviations: AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase; TUNEL: Terminal Deoxyribonucleotidyl Transferase Mediated Nick End Labeling; OP: Organophosphorus Compounds

Introduction

Cocaine is one of the most frequently abused illicit drugs worldwide. Its consumption is known to cause addictive dependence and is responsible for significant numbers of emergency room visits [1]. The use of cocaine has increased in this century, being one of the major public health problems [2]. Cocaine is toxic to the central nervous system (CNS) and the cardiovascular system [2-4] and causes liver injury in human and animals [5-7]. Cocaine use has been associated with hepatotoxicity in human beings, which was documented by biochemical analysis, tomography and biopsy [8,9]. Growing evidences indicated that the hepatotoxicity induced by cocaine is related to apoptosis of hepatocytes [10-12]. It is imperative to reveal the underlying mechanisms of cocaine-induced apoptosis related to its toxic effects.

Acetylcholinesterase (AChE) is well known for its function to rapidly hydrolyze acetylcholine into acetate and choline. It also has some noncholinergic functions including the control of cell apoptosis [13,14]. Cultured cell lines acquired AChE expression when subjected to conditions that induced apoptosis [13,15,16]. We reported previously that plasma AChE activity was induced after inhibition initially in mice treated with organophosphorus poisons (OP) [17,18] and induced in neuronal cells and mice treated with ethanol [19]. Research is scarce regarding the role of AChE on cocaine-induced apoptosis.

In the present work carboxylesterase and butyrylcholinesterase knockout ES1-/-BChE-/- mice were used as models because humans have no plasma carboxylesterase [20]. Since wild-type mouse liver has very little AChE activity and huge amounts of BChE activity, use of butyrylcholinesterase deficient mice in the cocaine challenge experiment eliminates the background BChE activity and therefore makes it possible to measure small changes in AChE activity.

The goal of this study was to determine whether cocaine, known to be an inducer of apoptosis [10,11], increased AChE activity in mice. We hypothesized that treatment with cocaine triggers an apoptotic cascade, which results in increased plasma AChE in living mice. We observed mouse plasma AChE activity increased after cocaine

exposure and a direct relationship between AChE activity and apoptosis in the liver of treated mice. We firstly reported that cocaine exposure induces both excess AChE activity and apoptosis in animals.

Materials and Methods

After registering with the United States Department of Justice Drug Enforcement Administration, (-)-Cocaine hydrochloride (cat# C5776) was purchased from Sigma-Aldrich (St. Louis, MO). Cocaine was dissolved in water to make a 0.1 M stock solution and stored at -80°C. Absolute ethyl alcohol was purchased from Aaper Alcohol & Chemical Co. (Shelbyville, KY). Tissue-Tek O.C.T. (cat# 4583) was purchased from Sakura Finetek Inc. (Torrance, CA). The In Situ Apoptosis Detection Kit #MK500 was from Clontech Laboratories Inc. (Madison, WI).

Mice

All animal work was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Formal approval to conduct the animal experiments was obtained from the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Mice in strain C57BL/6 were bred from parental ES1-/- plasma carboxylesterase knockouts [17] and BChE-/- butyrylcholinesterase knockouts [21] from the Jackson laboratory to produce the ES1-/-BChE-/- genotypes at University of Nebraska Medical Center. The plasma carboxylesterase knockout mice are listed as JAX Stock No. 014096 strain name C57BL/6-Ces1c$^{tm1.1Loc}$/J and the butyrylcholinesterase knockout mice are listed as JAX Stock No. 008087 strain name B6.129S1-Bche^{tm1Loc}/J.

Adult ES1-/-BChE-/- mice had no plasma carboxylesterase activity, no BChE activity in plasma and tissues, and were healthy and fertile with normal AChE activity. The mice used in this work were at least 6 months old with an average weight of 25 g. Mouse bedding was changed daily.

Challenge with cocaine

All mice were housed singly to avoid stress from post treatment aggressive behavior. Pre-dose body weights and surface body temperatures were recorded for each mouse. Adult female ES1-/-BChE-/- mice (n=5 for each group) were treated intraperitoneally daily at 10 AM for 8 days with either water as control or 25 mg/kg cocaine. The treatment was done in the laboratory at room temperature. This dose was chosen based on our previous work which showed that mice chronically treated with a similar dose had hepatotoxicity and cardiotoxicity, but no mortality [17]. Blood (50 µl) was collected from the saphenous vein prior to dosing and on the ninth day before sacrifice for measurement of plasma AChE activity. Animals were sacrificed on day 9 by inhalation of carbon dioxide and then perfused via intracardial with 50 ml of 0.1 M phosphate-buffered saline (PBS) to wash out blood. Brain, heart, kidney, and liver were homogenized on day 9 and tested for AChE activity. BChE activity was not tested because the mice had no BChE activity [21].

Functional observational battery

Physiological and behavioral changes were observed and recorded before and after dosing to assess potentially adverse effects as reported by McDaniel and Moser [22]. The test battery includes following symptoms: changes in posture, involuntary motor movements, tremors, palpebral closure, lacrimation, salivation, gait, arousal, hyperactivity, delayed righting reflex, etc.

Temperature measurement

Axial body temperature of mice was measured using a digital thermometer (Thermalert model TH-5) attached to a surface Microprobe MT-D, type T thermocouple (Physitemp Instruments, Clifton, NJ). Surface body temperature was recorded prior to dosing, as well as every 10 minute for 1 hour, and every 30 min through 3 h post dosing.

AChE activity measurement

AChE activity in plasma and selected tissues was measured with 1 mM acetylthiocholine and 0.5 mM dithiobisnitrobenzoic acid in potassium phosphate pH 7.0 at 25°C [19]. Three µl of plasma or 50 µl of tissue homogenate supernatant were used for each sample in a total 2 ml reaction volume. The increase in absorbance at 412 nm was recorded by spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) interfaced to MacLab 200 (ADInstruments, Pty Ltd., Castle Hill, Australia) and a Macintosh computer. Activity was calculated using the extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for the product with yellow color. A unit of activity was defined as 1 µmol of substrate hydrolyzed per minute.

Nondenaturing gradient gel electrophoresis and staining for AChE activity

Gradient (4-30%) polyacrylamide gels (0.75 mm thick, 15 × 11 cm) were cast in a Hoefer SE600 gel apparatus (Hoefer, Holliston, MA). For each lane 7.5 µl of mouse plasma was used with an equal volume of 0.1 M TrisCl pH 6.8, 50% glycerol, 0.1% bromophenol blue. Plasma from AChE-/- mice contained only BChE activity and no AChE activity [23]. Plasma from BChE-/- mice contained only AChE activity and no BChE activity [21]. Electrophoresis was conducted at a constant voltage (250 V) at 4°C for 20 h. Gels were stained for cholinesterase activity by the modified method of Karnovsky and Roots [24].

Tissue sections

Tissues from control mice and mice treated with cocaine were prepared for sectioning. Brain, heart, liver, and kidney were embedded in O.C.T. medium on dry ice for frozen section. The embedded tissues were then sliced into 10 µm sections onto silanized slides.

Terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling (TUNEL)

Apoptotic DNA fragments were detected by the In Situ Apoptosis Detection kit. Sections were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, washed twice with PBS, and then permeabilized with permeabilization buffer for 5 minutes on ice before incubation with the TUNEL reaction mixture for three hours at 37°C. Apoptotic cells were detected by a Nikon Eclipse E800 fluorescence microscope using a fluorescent light source as described before [19]. TUNEL positive cells were counted in 6 randomly chosen fields at 100X magnification. The numbers were compared between the cocaine treatment and control groups.

Double staining for AChE activity and TUNEL

The modified method for AChE activity staining is described by Karnovsky and Roots [24]. Slides were incubated for 2 h with 3 mM copper sulfate, 0.12 M maleic acid pH 6 containing 5 mM sodium citrate, 2 mM acetylthiocholine, and 0.5 mM potassium ferricyanide and then washed with 50 mM Tris-HCl pH 7.4. AChE positive areas with dark brown color was visualized by microscopy using a bright field source. The sections were counterstained with fluorescent dUTP to detect DNA breaks. Cells stained for TUNEL were detected by a fluorescence microscope (Nikon Eclipse E800).

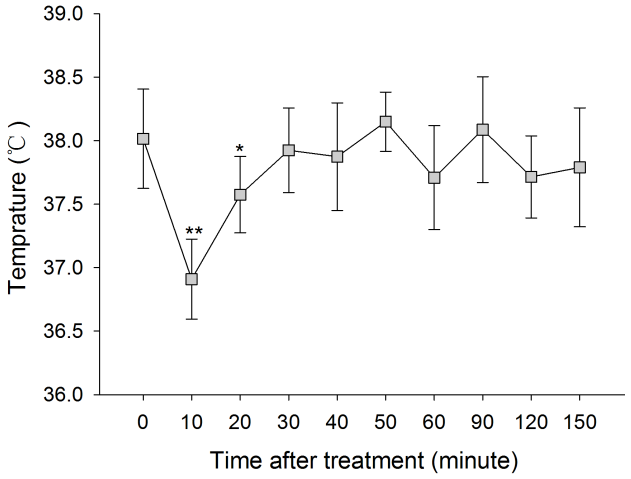

Figure 1: Eight-day averaged surface body temperature (±S.D.) of ES1-/-BChE-/- mice treated daily with 25 mg/kg cocaine. *as compared with control temperature before treatment, P<0.05, ** as compared with control, P<0.01.

Statistical analysis

Results were expressed as mean values ± standard deviation. The One-Way ANOVA was performed to evaluate statistical significance of differences. The level of statistical significance was set at p<0.05. The analysis used SPSS 16.0 software (Microsoft Corp., Redmond, WA).

Results

Toxicity in mice following cocaine treatment

Mice treated daily for 8 days with cocaine (25 mg/kg, intraperitoneally) were hyperactive up to 3 h post-dosing, determined by counting the number of times the mouse crossed a line on the cage. Mice lost 8.8% of their pre-dose body weight during the days of treatment. Control groups had no weight loss during the 9-day trial. Surface body temperature had a 1°C decrease at 10 min after treatment then returned to base line by 30 min (Figure 1). Water-treated control mice maintained normal body temperature throughout the treatment period.

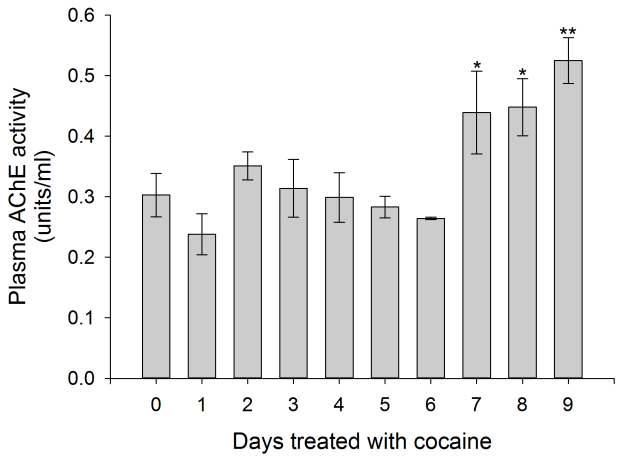

Figure 2: Plasma AChE activity on days 0-9 in ES1-/-BChE-/- mice treated with cocaine 25 mg/kg daily for 8 days. Plasma AChE activity on days 7-9 in the cocaine-treated group was significantly elevated, as compared with control activity before treatment at day 0. Error bars are ± standard deviation. Animals had the genotype ES1-/-BChE-/-, which means they had no plasma carboxylesterase and no butyrylcholinesterase. *P<0.05, **P<0.01 as compared with control activities. N=5 for each group.

Excess plasma AChE activity in mice treated with cocaine

Mice were treated with cocaine at a dose and schedule that has previously been shown to cause hepatotoxicity and apoptosis [25]. Plasma samples from mice challenged intraperitoneally with 25 mg/kg cocaine daily for 8 days were measured for AChE activity (Figure 2) and analyzed on a nondenaturing gradient gel (Figure 3). Plasma AChE activity increased from 0.3 unit/ml to 0.44 unit/ml on day 7 and remained elevated on days 8 and 9. The induced AChE activity was visualized on a nondenaturing gel where the day 9 plasma samples had a broad, fuzzy band that migrated in row 3 (Figure 3). The position of the induced AChE activity is similar to the position of the OP induced AChE activity [18], suggesting that cocaine induced similar AChE forms as OP. Perfused brain, heart, kidney and liver tissues from cocaine treated mice were homogenized and tested for AChE activity. No significant difference in AChE activity was observed compared to controls. The animals in the cocaine experiment were completely deficient in butyrylcholinesterase, therefore BChE activity was not measured.

Increased apoptosis in liver of mice exposed to cocaine on day 9

Apoptosis was evaluated by counting TUNEL positive cells in control and cocaine treated mouse tissues. Figure 4 shows that the number of apoptotic cells in liver sections from treated mice on day 9 increased about 2-fold compared to control mice (P<0.05). There was no significant increase in the number of apoptotic cells in brain, heart, and kidney sections.

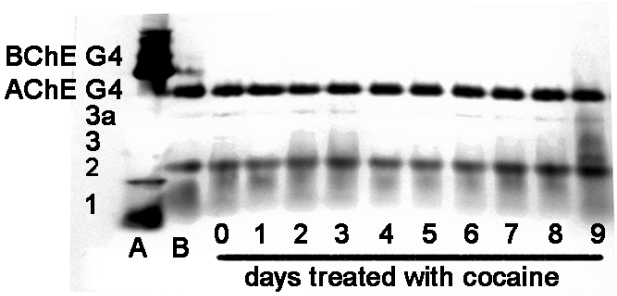

Figure 3: Visualization of plasma AChE activity on a non-denaturing gel stained with acetylthiocholine after ES1-/-BChE-/- mice were challenged with cocaine. The gel shows the molecular forms of AChE in the plasma samples. Lane A): plasma from AChE knockout mouse demonstrating BChE bands. The heavy bands above the main BChE tetramer band (G4) are aggregates formed during the storage of plasma. Lane B): plasma from BChE knockout mouse demonstrating AChE bands. The row of bands labeled G4 designates tetrameric AChE. Monomeric AChE migrates in a diffuse band at the front of the gel in position 1. Dimeric and trimeric AChE bands are shown in positions 2, 3 and 3a. The induced AChE activity mostly appears in rows 3a and 3.

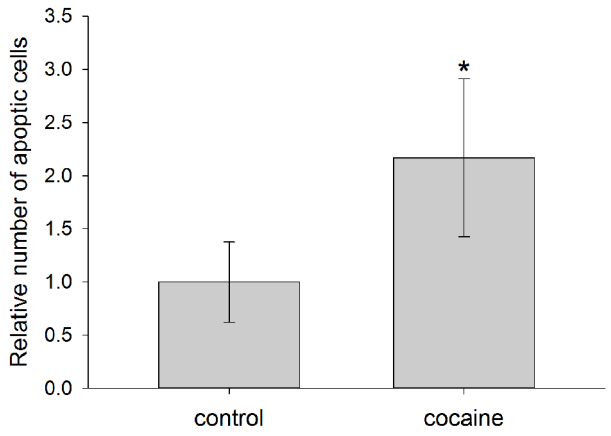

Figure 4: Quantification of apoptotic cells in liver sections from mice exposed to cocaine compared to control mice on day 9. Cells with green fluorescent nuclei were TUNEL-positive cells. Six randomly chosen fields were observed for each mouse. *As compared to control, P<0.05. N=5 for each group.

AChE activity was associated with apoptosis

Mouse tissue sections were stained both for AChE activity and DNA fragmentation to investigate whether there is an association between AChE activity and apoptosis in tissues from treated mice. As shown in Figure 5, liver cells that stained heavily for AChE activity were also brightly fluorescent for DNA fragmentation, which demonstrated that apoptotic cells are associated with unusual high AChE activity. After cocaine exposure an excess of AChE activity was detected in mouse plasma. It is reasonable to assume that the excess AChE in plasma might be from apoptotic cells.

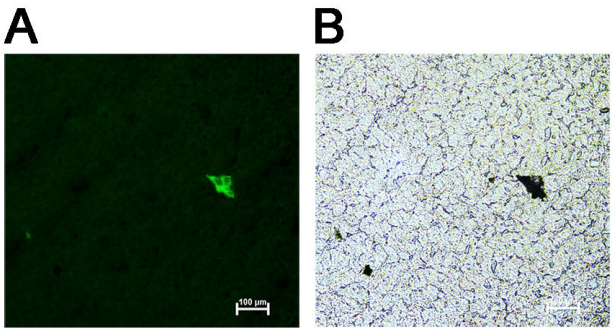

Figure 5: Double staining of liver sections for AChE activity and TUNEL assay. The sections were from mice challenged intraperitoneally with 25 mg/kg cocaine every day and sacrificed on day 9. Sections in the left column (Figure 5A) were stained for DNA fragmentation with the TUNEL assay. The bright green fluorescent areas are positive for genomic DNA fragmentation. The same sections, in the right column (Figure 5B), were stained for AChE activity. The dark brown areas are positive for AChE activity. Scale bar: 100 µm.

Discussion

Our previous work showed that mice treated with OP had excess plasma AChE activity above base line after inhibition [17,18]. In the present study, mice were treated with cocaine, a drug that does not inhibit AChE activity like OP, but causes apoptosis in cultured cells and animals [26,27]. The chemical structure of cocaine (Figure 6) shows that cocaine is an uncharged, lipophilic diester, which allows for its rapid absorption through nasal membranes and crossing the blood-brain barrier [28].

ES1-/-BChE-/-mice used for cocaine research

Butyrylcholinesterase hydrolyzes cocaine and protects from cocaine hepatotoxicity [29-31]. Cocaine detoxification is primarily by BChE in human beings [32]. One out of 4 humans has a BChE deficient genotype and genetic variants result in altered catalytic activity [33]. The BChE knockout mice had more severe hepatotoxicity than the wild type mice and they are important animal models for studying pathological effects of cocaine [25]. Humans have no carboxylesterase in plasma [20].

In the present work, the plasma carboxylesterase and butyrylcholinesterase knockout mouse model (ES1-/-BChE-/-) was used to provide an in vivo tool for studying drug sensitivity [34]. The use of mice that were completely deficient in BChE made it more sensitive to monitor the change of AChE activity. In previous reports, we used ES1-/-BChE+/- and ES1-/-BChE+/+ mice as well as wild-type mice to study induced AChE activity [17,18]. All mice with different genotypes responded to apoptosis-inducer treatment with excess plasma AChE activity, which suggests that plasma carboxylesterase and butyrylcholinesterase may not be responsible for induction of AChE activity in mouse plasma.

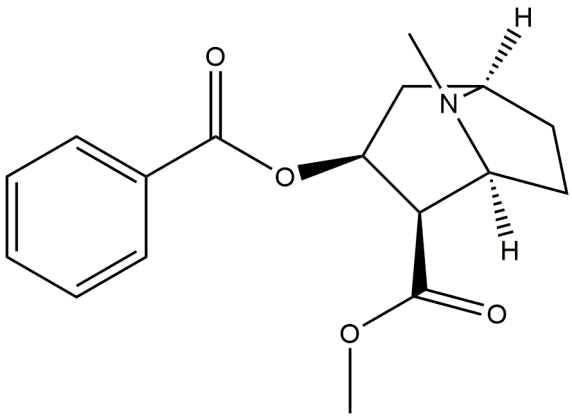

Figure 6: The structure of cocaine (Formula: C17H21NO4) was drawn with ChemDraw 14.0 software (CambridgeSoft Corporation, Cambridge, MA).

High-dose cocaine use can result in high body temperature and cause life-threatening hyperthermia in humans. The ability to raise core temperature is one of the most dangerous effects of cocaine toxicity [35,36]. We observed that cocaine administration caused the BChE–/– mice to become more hypothermic than the control group, which is actually opposite to the results from cocaine treated BChE+/+ mice. Wild type mice were hyperthermic after dosing in our previous work [34]. It is possible that cocaine may cause the release of noradrenaline centrally or have a direct role on regulating the thermoregulatory center in the hypothalamus [37].

Excess plasma AChE is a biomarker of cocaine toxicity

AChE has been reported to play an important role in apoptosis in various cell types. It is demonstrated that cocaine induces apoptosis in mouse tissues [26,38]. However, whether increased plasma AChE is associated with cocaine-induced apoptosis in mice was unknown. We found AChE activity in the plasma of cocaine treated mice was elevated significantly above normal level. It was established that cocaine treated mice had more apoptotic cells in liver tissue compared to untreated control mice, and excess AChE activity is associated with apoptotic cells.

Cocaine could cause hepatotoxicity, suggesting that apoptotic liver cells may release AChE into the circulation and be one source of the excess plasma AChE. No change in AChE activity was observed in homogenates of whole liver. Therefore, general liver damage is not the source of the increased plasma AChE activity.

Roles of AChE in cocaine-induced toxicity

Cocaine is primarily metabolized in the liver. Mechanisms of cocaine-induced liver toxicity have been studied extensively and most of the studies have focused on the bioactivation of cocaine, disruption of redox homeostasis, and oxidative stress [28,39,40]. In apoptotic cells, AChE is expressed and necessary for apoptosome assembly and caspase-9 activation [41].

Acetylcholinesterase inhibitor donepezil, huperzine A or rivastigmine were reported to attenuate cocaine-induced toxic symptoms for cocaine use disorder [42-44]. Elevated levels of plasma AChE activity may play a role in the pathogenesis of some neurodegenerative diseases [45]. The fact that cocaine induced plasma AChE level might be related to cocaine neurotoxicity [46]. The exact mechanism for the role of AChE in cocaine-induced toxicity is still unclear. It should be of interest to study how AChE contributes to cocaine toxicity and explore the underlying mechanisms.

Conclusion

Induction of plasma AChE activity correlates with increased apoptotic cells in mice treated with cocaine. It is concluded that increased plasma AChE activity may be an indicator for apoptosis in mice exposed to cocaine. However, the exact role of AChE in cocaine-induced toxicity is still not clear and warrants further study.

Ethics Approval

Formal approval to conduct the animal experiments was obtained from the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgement

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