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Study of acetylcholinesterase activity and apoptosis in SH-SY5Y cells and mice exposed to ethanol

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ABSTRACT

Ethanol is one of the most commonly abused psychotropic substances with deleterious effects on the central nervous system. Ethanol exposure during development results in the loss of neurons in brain regions and when exposed to ethanol cultured cells undergo apoptosis. To date no information is available on whether abnormally high AChE activity is characteristic of apoptosis in animals exposed to ethanol. The aims of the present study were to determine whether induction of AChE activity is associated with ethanol-induced apoptosis and to explore the mechanism of enhanced AChE activity induced by ethanol.

For this purpose, *in vitro* and *in vivo* experiments were performed. AChE activity was quantified by spectrophotometry and apoptosis by flow cytometer in SH-SY5Y cells exposed to ethanol. The results showed that cells treated with 500 mM ethanol for 24 h had a 9-fold increase in apoptotic cells and a 6-fold increase in AChE activity compared with controls. Mice exposed acutely to 200 μl of 20% ethanol daily on days 1–4 had elevated AChE activity in plasma on days 3–7. On day 4, plasma AChE activity was 2.4-fold higher than pretreatment activity. More apoptotic cells were found in the brains of treated mice compared to controls. Cells in brain sections that were positive in the TUNEL assay stained for AChE activity.

In conclusion, AChE activity and apoptosis were induced in SH-SY5Y cells and mice treated with ethanol, which may indicate that increased AChE may related to apoptosis induced by ethanol. Unusually high AChE activity may be an effect marker of exposure to ethanol. The relationship between AChE and apoptosis might represent a novel mechanism of ethanol-associated neuronal injury.

1. Introduction

Ethanol, also called alcohol, is a widely consumed beverage, which has deleterious effects on most organs of the body when overdosed. The central nervous system (CNS) is a particular target for the damaging consequences and several neurological disorders are related to excessive ethanol exposure (Moonat et al., 2010; Natarajan et al., 2015). Acute ethanol administration of animals selectively impairs learning and memory (Berry and Matthews, 2004; Ryabinin, 1998). Growing evidence indicates that the neurotoxicity induced by ethanol is related to apoptosis and necrosis (Guadagnoli et al., 2016; Heaton et al., 2003; Liesi, 1997). Neuronal loss in the brain in both fetuses and adults is a devastating consequence and is associated with mental retardation and other behavioral deficits (Brust, 2010; Elibol-Can et al., 2011; Ikonomidou et al., 2000). It is imperative for us to explore the underlying mechanisms of ethanol-induced neuronal apoptosis in order to develop therapeutic approaches to ameliorate ethanol neurotoxicity. Ethanol is the major constituent and a potent toxin in commonly consumed alcohols and is undoubtedly responsible for many of the effects of consumption. However, it is important to point out that impurities in those beverages can cause some of the symptoms. For example, headache resulting from drinking red wine is thought to be due to histamine impurities in the wine (Jarisch and Wantke, 1996). In this study, we focus on the ethanol induced effects and use absolute ethyl alcohol to explore the underlying mechanism.

Acetylcholinesterase (AChE) is well-known as the enzyme that

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; iso-OMPA, tetraisopropyl pyrophosphoramide; TUNEL, terminal deoxyribonucleotidyl transferase mediated nick end labeling

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rapidly splits acetylcholine into acetate and choline. AChE has been found to have noncholinergic functions including an association with cell apoptosis (Zhang and Greenberg, 2012). Zhang et al. found that cultured cell lines acquired AChE activity and AChE mRNA when subjected to conditions that stimulated apoptosis (Zhang et al., 2002). More than 40 different types of cells have shown AChE expression during apoptosis (Aluigi et al., 2010; Jing et al., 2008; Perez-Aguilar et al., 2015; Ye et al., 2015; Zhang and Greenberg, 2012). An unexpected enzymatic activity as DNase for synaptic acetylcholinesterase (AChE-S) was reported after cytotoxic insults (Du et al., 2015; Sanchez-Osuna and Yuste, 2015).

We demonstrated previously that plasma AChE activity was initially inhibited but then induced after 1–25 days in mice treated with organophosphorus poisons (Duysen and Lockridge, 2011). Induction of AChE activity in plasma and brain has been reported by other groups in rats exposed to organophosphorus agents (Bansal et al., 2009; Trevisan et al., 2008). Information about the effects of AChE on ethanol-induced apoptosis is scarce. Cholinesterase inhibitors were reported to attenuate spatial memory and cognitive flexibility impairment induced by acute ethanol exposure in rats (Gawel et al., 2016). We hypothesize that mice treated with acute dose of ethanol experience an apoptotic cascade resulting in increased levels of plasma AChE. The goal of present study was to determine whether apoptosis induced by ethanol is associated with increased AChE activity. The studies were initiated to explore the involvement of AChE in ethanol-induced neuronal apoptosis of SHSY5Y cells (human neuroblastoma cell line). Simultaneously, the hypothesis that AChE activity in living mice treated with ethanol increased and was associated with apoptosis was tested and a general increase in mouse plasma AChE activity upon exposure to ethanol was observed. A direct relationship between apoptosis and increased AChE activity in the brain of ethanol-treated mice was found. This is the first report to show that ethanol exposure induces excess AChE activity and apoptosis in animals.

2. Materials and methods

2.1. Materials

Absolute ethyl alcohol 200 proof was from Aaper Alcohol & Chemical Co., (Shelbyville, KY). Ethopropazine hydrochloride (cat# E-2880) and tetraisopropyl pyrophosphoramide (cat# T-1505) were purchased from Sigma (St.Louis,MO). Lithium heparin microvettes 300 LH were from Sarstedt (Newton, NC). Tissue-Tek O.C.T. (Optimal Cutting Temperature compound cat# 4583) was from Sakura Finetek Inc. (Torrance, CA). The In Situ Apoptosis Detection Kit #MK500 by TAKARA BIO INC. was purchased from Clontech Laboratories Inc. (Madison, WI).

2.2. Mice

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 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 at the University of Nebraska Medical Center from parental ES1 −/− plasma carboxylesterase knockouts (Duysen and Lockridge, 2011) and BChE −/− butyrylcholinesterase knockouts (Li et al., 2008) to produce the ES1 −/− BChE +/+, ES1 −/− BChE +/−, and ES1 −/− BChE −/− genotypes. The ES1 −/− mice are completely deficient in plasma carboxylesterase activity, but have normal carboxylesterase activity in liver, intestine, and other tissues. The butyrylcholinesterase deficiency reduces butyrylcholinesterase activity in all tissues. Adult ES1 −/− BChE +/+ mice had wild type levels of butyrylcholinesterase activity, whereas ES1 −/− BChE +/− mice had approximately one half of wild type levels, and ES1 −/− BChE −/− mice had no BChE activity. The mice are healthy and fertile. All mice had normal AChE activity. In the present work ES1 −/− BChE +/+ mice were used as models for humans because humans have no plasma carboxylesterase (Li et al., 2005). Mouse bedding was changed daily to minimize the effect of coprophagic behavior.

2.3. Methods

2.3.1. Cell culture and treatment

Human neuroblastoma SH-SY5Y cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium and HAM's F12 (hyclone, GE healthcare) supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 IU/ml) at 37 °C in a humidified incubator with 5% CO_2 atmosphere. Cells were allowed to adhere to the bottom surface of a 60 mm culture dish for 24 h before treatment with fresh medium containing 0, 50, 100, 200, 500 mM ethanol for 24 h. The ethanol for cell studies was 200 proof (17.1 M). Cells treated with 0.05% hydrogen peroxide served as a positive control for apoptosis. For all cell studies 5 replicate experiments were performed.

2.3.2. Determination of AChE activity in cells

AChE activity was measured spectrophotometrically in a 96-well microtiter plate according to a modified Ellman assay (Ellman et al., 1961; Santillo and Liu, 2015). Viable SH-SY5Y cells were trypsinized and then washed with cold PBS to remove culture medium before they were incubated at room temperature with a reaction mixture (0.2 ml) containing DTNB (0.5 mM) and ATCh (1 mM). Absorbance of the product TNB (λ = 410 nm) was measured every 10 min up to 60 min, and the slopes of absorbance *vs.* time curves were equal to activity. The experiments were performed using 20000 SH-SY5Y cells per well in 96-well plates. Activity of incubations without substrate was subtracted as background.

2.3.3. Flow cytometry analysis of cell apoptosis

Apoptosis was assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (lot # 5300148, Multisciences, Hangzhou, China) by flow cytometry (Beckman Coulter, CA, USA). After treatment, SH-SY5Y cells were trypsinized, washed with cold PBS, and re-suspended in 500 μl binding buffer containing 5 μl Annexin-V-FITC and 10 μl propidium iodide (PI). After incubation for 15 min at 37 °C in the dark, fluorescence levels were quantified by flow cytometry.

2.4. Mouse work

2.4.1. Challenge with ethanol

Adult female ES1 −/− BChE +/+ mice (n = 10) were divided into ethanol group and control group. The ethanol group (n = 5) was treated intraperitoneally with 200 μl of a 20% solution of ethanol in water (Jana et al., 2010) daily on days 1–4. This is a dose of approximately 175 mg/dl per day (Plackett and Kovacs, 2008). Animals were sacrificed on day 7 after treatment. The control group (n = 5) was treated with water. Blood (50 μl) from the treatment and control groups was collected from the saphenous vein into heparinized tubes each day for measurement of AChE activity in plasma. Mice were perfused to wash out blood before tissues were collected. Spleen, stomach, lung, heart, kidney, quadriceps muscle, liver and brain were homogenized and assayed for AChE activity.

2.4.2. Functional observational battery

The behavioral toxic signs of mice including posture, involuntary motor movements, tremors, seizures, convulsions, palpebral closure, reactivity to being handled, lacrimation, salivation, piloerection, gait, mobility, arousal, stereotypy, straub tail, vocalization, righting reflex, and hyperactivity were observed according to description by McDaniel and Moser (McDaniel and Moser, 1993).

2.4.3. Temperature measurement

Axial body temperature was measured with a digital thermometer, Thermalert model TH-5, attached to a surface Microprobe MT-D, type T thermocouple (Physitemp Instruments, Clifton, NJ). Temperature was recorded prior to challenge, at 5 min intervals for the first hour post-challenge, hourly through 8 h, and daily post dosing.

2.4.4. Enzyme activity measurement

AChE activity in mouse plasma and selected tissues was assayed with 1 mM acetylthiocholine in 0.1 M potassium phosphate pH 7.0, 0.5 mM dithiobisnitrobenzoic acid (DTNB), in the presence of 0.01 mM ethopropazine to inhibit BChE. BChE activity was assayed with 1 mM butyrylthiocholine in 0.1 M potassium phosphate pH 7.0, 0.5 mM dithiobisnitrobenzoic acid. Both assays were conducted at 25 °C. Three µl of plasma or 50 µl of clarified tissue homogenate supernatant was used in a 2 ml reaction volume in quartz cuvettes. The change in absorbance at 412 nm was recorded on a temperature-controlled spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) interfaced to MacLab 200 (ADInstruments, Pty Ltd., Castle Hill, Australia) and a Macintosh computer. Each sample was repeated for three times in activity measurement. Activity was calculated in micromoles per minute from the extinction coefficient of 13,600 M^{-1} cm^{-1}. A unit of AChE or BChE activity was defined as one micromole of substrate hydrolyzed per minute.

2.4.5. Nondenaturing gradient gel electrophoresis and staining for AChE activity

Gradient polyacrylamide gels (4–30%) were cast in a Hoefer SE600 gel apparatus (Hoefer, Holliston, MA) to make 15 × 11 cm gels, 0.75 mm thick. Plasma samples from control and treated mice were incubated with 0.1 mM iso-OMPA for 40 min to inhibit BChE. Following inhibition the plasma samples (7.5 µl per lane) were mixed with an equal volume of 50% glycerol, 0.1 M TrisCl pH 6.8, 0.1% bromophenol blue before loading on the gel. Mouse plasma that contained only BChE activity and no AChE activity was from AChE −/− mice (Xie et al., 2000). Mouse plasma that contained only AChE activity and no BChE activity was from BChE −/− mice (Li et al., 2008). Electrophoresis was conducted at 250 V constant voltage for 20 h at 4 °C. Gels were soaked in 0.1 mM iso-OMPA in water for 30 min before they were stained for AChE activity by the method of Karnovsky and Roots (Karnovsky and Roots, 1964). The staining solution contained 180 ml of 0.2 M maleic acid adjusted to pH 6 with 1 M NaOH, 30 ml of 0.03 M $CuSO_4$, 30 ml of 5 mM potassium ferricyanide, 15 ml of 0.1 M sodium citrate, 30 ml water and 150 mg of acetylthiocholine iodide. The purpose of pretreating gels with iso-OMPA was to inhibit BChE. Since BChE hydrolyzes acetylthiocholine, reducing the intensity of BChE bands with iso-OMPA allowed better visualization of AChE bands. Gels were incubated overnight with gentle shaking. The reaction was stopped by washing the gels with water.

2.4.6. Tissue sections

Tissues from mice treated with ethanol and from control mice were prepared for sectioning (n = 5 each group). Lungs were removed gently and inflated with Tissue-Tek O.C.T. through a tracheal cannula (Myung et al., 2008). The lung (inflated), heart, liver, brain, abdominal fat, quadriceps muscle, spleen, testes, kidney, and small intestine were embedded in O.C.T. compound on dry ice. The frozen tissues were sliced into 10 µm sections with a cryostat stick (Leica CM 1850) onto silanized slides. Apoptotic cells were identified by labeling the DNA breaks with fluorescent-tagged deoxyuridine triphosphate nucleotides in the TUNEL assay described below, and by staining for AChE activity.

2.4.7. Double staining for AChE activity and TUNEL

The method for AChE activity staining is described by Karnovsky and Roots (Karnovsky and Roots, 1964). All reagents were filtered through a 0.45 µm filter to remove particulates. Slides in an 8-slide glass staining jar were washed three times with 0.1 M maleic acid pH 6.0 and incubated in 1 mM iso-OMPA for 40 min to inhibit BChE. The iso-OMPA containing buffer was poured off and replaced with 75 ml of 0.12 M maleic acid pH 6 containing 5 mM sodium citrate, 3 mM copper sulfate, 0.5 mM potassium ferricyanide, and 2 mM acetylthiocholine. After 2 h incubation at room temperature, the slides were washed 3 times with 50 mM Tris-HCl pH 7.4. The reddish brown color of AChE positive areas was visualized with a Nikon Eclipse E800 microscope using a bright field source. The slides were counterstained for DNA breaks with fluorescent dUTP in a TUNEL assay. Images of cells stained for TUNEL were captured by a camera mounted on the Nikon Eclipse E800 microscope, using a fluorescent light source. The fluorescent light source consisted of an excitation band pass filter that provided 465–495 nm excitation light and an emission band pass filter that transmitted fluorescence at 515–555 nm fluorescence emission.

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

DNA fragments resulting from the apoptotic activation of intracellular endonucleases were detected in tissue sections by incorporation of fluorescein labeled dUTP using the In Situ Apoptosis Detection kit from Clontech, as described by the manufacturer. Briefly, sections were fixed in 4% paraformaldehyde for 30 min, washed twice with phosphate buffered saline, and permeabilized with permeabilization buffer for 5 min on ice. Sections were incubated with the TUNEL reaction mixture (consisting of Terminal deoxynucleotidyl-transferase enzyme and Labeling Safe Buffer) for three hours in a 37 °C humidified incubator. The labeling reaction was terminated by washing 3 times with phosphate buffered saline. Apoptotic cells were detected by fluorescence microscopy (Nikon, Eclipse E800) as described in the methods section on double staining for AChE activity and TUNEL. TUNEL positive cells were counted in 6 randomly selected fields at 100× magnification. Findings were compared between the ethanol and control groups.

2.5. Statistical analysis

The results were expressed as mean values ± standard deviation. The One-Way ANOVA was used to evaluate statistical significance by SPSS 16.0 software. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Ethanol induced apoptosis in SH-SY5Y cells

Apoptosis in SH-SY5Y cells treated with various concentrations of ethanol (50–500 mM) for 24 h and control cells was measured by flow cytometry. Fig. 1 showed the effects of various concentrations of ethanol on SH-SY5Y cell apoptosis. There were no significant effects of ethanol on cell apoptosis up to 100 mM ethanol compared to control. At higher concentrations, there was a concentration-dependent toxicity where about 25% of the cells were apoptotic at 500 mM ethanol. Exposure to ethanol for 24 h induced early and late apoptosis up to 2.2 and 9.3 folds at 200 and 500 mM ethanol, respectively.

3.2. Cells treated with ethanol have induced AChE activity

Fig. 2 shows the increased levels of AChE activity in SH-SY5Y cells exposed to 50 to 500 mM ethanol for 24 h. Ethanol treatment for 24 h at 200 mM and 500 mM concentration induced AChE activity up to 6-fold compared to control cells. There was no significant induction up to 100 mM ethanol, consistent with the results of ethanol-induced apoptosis in cells.

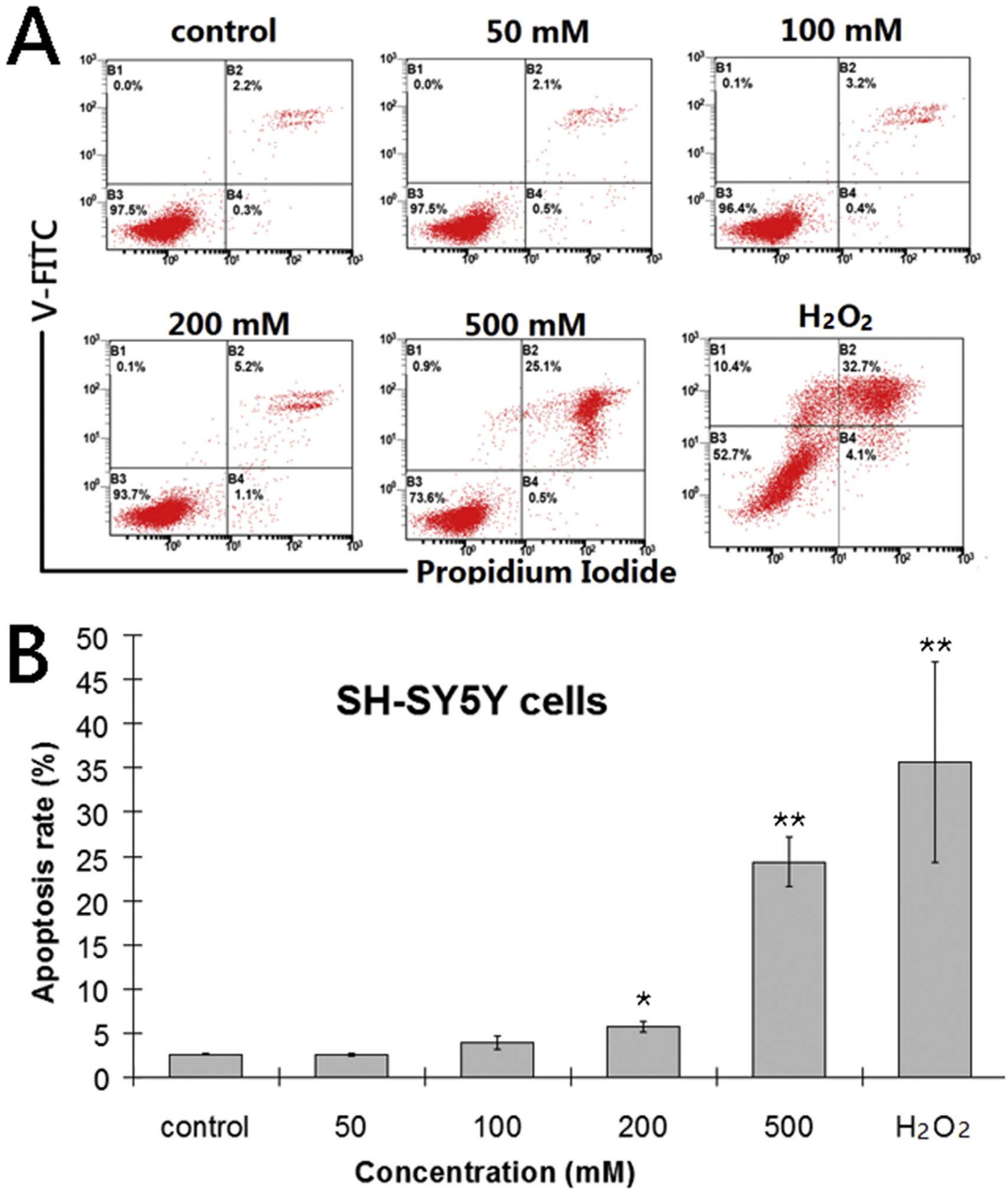

Fig. 1. The apoptosis detection of SHSY5Y cells exposed to ethanol. Fig. 1A shows apoptosis of SH-SY5Y cells exposed to ethanol for 24 h analyzed by Annexin V-FITC/PI staining with flow cytometry analysis. Data are presented as dot plots. (B1): quadrant I (necrotic cells); (B2): quadrant 2 (early apoptotic cells); (B3): quadrant 3 (living cells); (B4): quadrant 4 (late apoptotic cells). Fig. 1B showed the apoptosis rates of SH-SY5Y cells exposed to ethanol at the different doses, control and positive control (0.05% v/v H_2O_2). *as compared with control $P < 0.05$, ** as compared with control $P < 0.01$.

3.3. Minimal toxic signs in mice treated with ethanol

Mice treated with ethanol (175 mg/dl ip) on days 1–4 appeared to be slightly sedated for the first 30 min after dosing, but had no other signs of toxicity. Their body temperature and weight remained normal.

3.4. Effects of ethanol on plasma AChE and BChE activities in mice

Plasma samples from mice treated intraperitoneally with 200 μl of a 20% ethanol solution daily on days 1–4 were measured for AChE and BChE activities (Fig. 3). Fig. 3A displays that AChE activity in the plasma of ethanol treated mice was elevated 2.4-fold above normal, increasing from 0.3 to 0.71 u/ml on day 4 of treatment. But there was no significant change in plasma BChE activity in ethanol treated mice among different days (Fig. 3B). AChE and BChE activities in the plasma of control mice both remained normal during the study period.

Plasma samples from mice treated with ethanol were collected before and after dosing and analyzed on a non-denaturing gradient polyacrylamide gel stained for activity to visualize the size of induced AChE. Fig. 4 demonstrated the induced AChE activity migrated in a broad, fuzzy band in row 3 and 3a between tetrameric AChE in row G4 and dimeric AChE in row 2, which suggest that induced AChE is smaller than AChE tetramer.

Mice had the genotype ES1 −/− BChE +/+ which means they had wild-type levels of butyrylcholinesterase activity. The nondenaturing gel in Fig. 4 shows BChE tetramer bands in all plasma samples despite the fact that the gel had been preincubated with 0.1 mM iso-OMPA to inhibit BChE. BChE hydrolyzes acetylthiocholine and is therefore

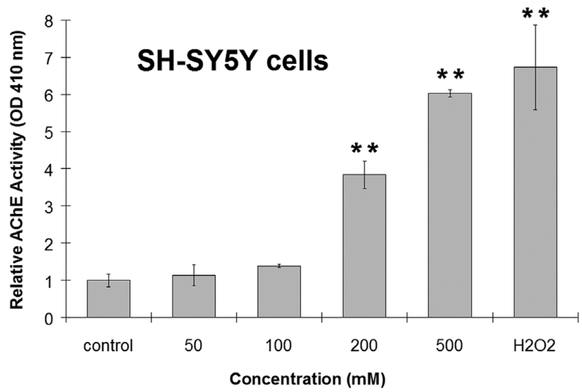

Fig. 2. The AChE activities in SH-SY5Y cells exposed to ethanol at 50–500 mM concentrations for 24 h. H_2O_2 (0.05% v/v) was served as positive control. ** As compared with control cells, $P < 0.01$.

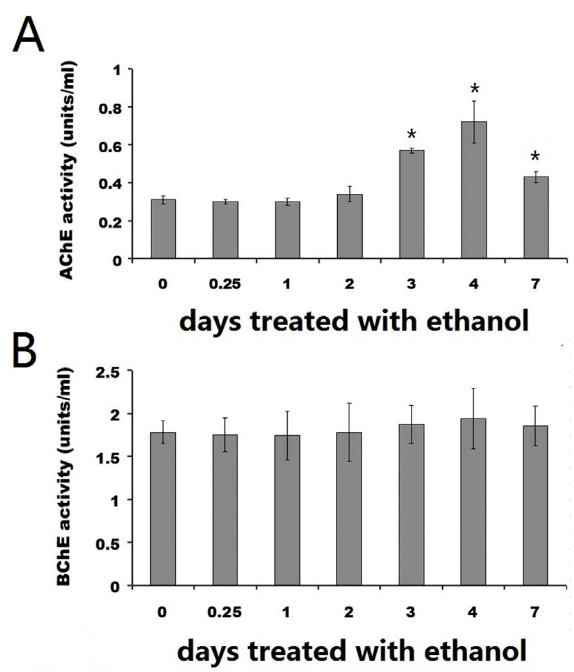

Fig. 3. The AChE and BChE activities in the plasma of mice exposed to ethanol for 0.25–7 days. Fig. 3A displays the AChE activity on days 3–7 in ethanol group significantly elevated, as compared with control activity before treatment at day 0 ($P < 0.05$). Fig. 3B displays the BChE activity in ethanol group did not significantly changed, as compared with day 0. Animals had the genotype ES1−/−BChE+/+. *As compared with control activities before treatment on day 0, $P < 0.05$. N = 5 for each group.

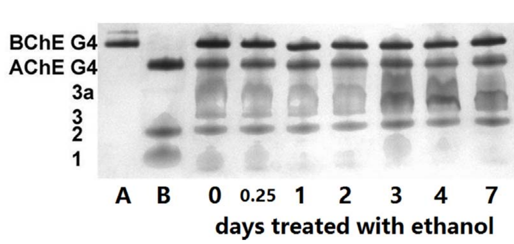

Fig. 4. Visualization of plasma AChE and BChE activities on a non-denaturing gel stained for activity with acetylthiocholine as a function of days after mice were challenged with ethanol. The gel shows the molecular forms of AChE in the plasma samples. Plasma was loaded at 7.5 µl/lane. Lane (A): plasma from AChE knockout mouse demonstrating a heavy band for BChE tetramers, labeled BChE G4. Lane (B): plasma from BChE knockout mouse demonstrating a heavy band for AChE tetramers, labeled AChE G4, and minor bands corresponding to AChE monomers (1) and dimers (2). The induced AChE activity appears as a fuzzy region in rows 3a and 3.

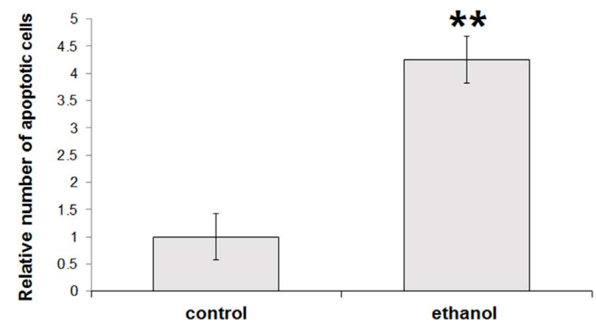

Fig. 5. Quantification of TUNEL-positive cells in brain sections from mice exposed to ethanol compared to control mice. Brains were obtained on day 7 after exposure to ethanol. Cells in brain sections with green fluorescent nuclei were TUNEL-positive cells observed in 6 randomly chosen fields for each mouse. ** As compared to control, $P < 0.01$. N = 5 for each group.

visible on gels stained for activity with acetylthiocholine.

3.5. Increased apoptosis in mice exposed to ethanol on day 7 after treatment

Apoptosis was measured by counting TUNEL positive nuclei as an indicator of apoptotic activity in control and ethanol treated mouse tissue sections. The TUNEL assay was performed on tissue sections from liver, spleen, heart, quadriceps muscle, testis, kidney, lung, and brain. Fig. 5 shows the number of apoptotic cells in brain sections from ethanol treated mice on day 7 after exposure significantly increased (about 4-fold), as compared to control mice ($P < 0.01$). There was no significant increase of the number of apoptotic cells in other tissues from ethanol group.

3.6. AChE activity was associated with apoptosis

It was of interest to determine whether apoptotic cells had high levels of AChE activity in mouse tissues. For this purpose, tissue sections were stained both for AChE activity and TUNEL staining of the same sections to identify genomic DNA fragmentation. As shown in Fig. 6, cells that stained heavily for AChE activity were also brightly fluorescent in the TUNEL assay. The brain sections are from ethanol treated mice. The sections showed cells that co-stained for AChE activity and for DNA fragmentation in the TUNEL assay. It is apparent that apoptotic cells are associated with high levels of AChE activity. That is, ethanol that induces apoptosis in the brains of a living mouse simultaneously induces excess AChE activity in apoptotic cells. After ethanol exposure an excess of AChE activity appears in mouse plasma. In light of the high levels of AChE activity that are observed in apoptotic cells, it is reasonable to propose that the excess plasma AChE might be produced by cells undergoing apoptosis.

4. Discussion

Our earlier work showed that mice treated with organophosphorus compounds (OP) had excess plasma AChE activity higher by 1.5 to 2.5 folds above normal (Duysen and Lockridge, 2011; Jiang et al., 2012). The induced AChE activity was observed despite the fact that OPs irreversibly inhibit acetylcholinesterase activity. In the present study, mice were treated with ethanol that does not inhibit AChE activity but is known to cause apoptosis in cultured cells and mice (Guadagnoli et al., 2016; Ren et al., 2016; Shim et al., 2016).

4.1. Ethanol could induce cell apoptosis together with AChE expression in cells

Ethanol treatment was reported to induce cell apoptosis by increasing ROS levels and endoplasmic reticulum stress (Ke et al., 2011; Liu

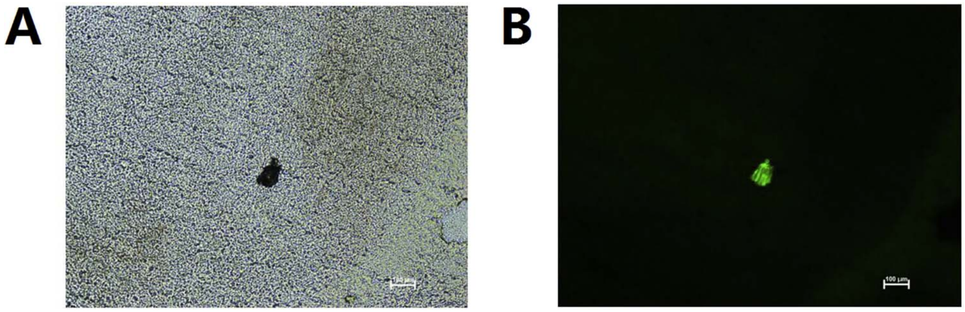

Fig. 6. Double staining for AChE activity and TUNEL assay. The sections of brains were from mice challenged intraperitoneally with 200 μl of a 20% ethanol solution everyday for 4 days and the mice were sacrificed on day 7 after exposure. Fig. 6A shows sections stained for AChE activity; the dark brown areas are positive for AChE activity. Fig. 6B in the right column shows the same sections stained for genomic DNA fragmentation by the TUNEL assay; the green fluorescent areas are positive for genomic DNA fragmentation. Scale bar, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2016; Shim et al., 2016; Yang and Luo, 2015). It was observed that SH-SY5Y cells were tolerant to low concentrations of ethanol (100 mM), but became apoptotic in response to high doses (500 mM) of ethanol. We found that 500 mM ethanol caused massive cell apoptosis in 24 h in the study. Similarly, there was no significant induction of AChE activity in cells treated with 100 mM or less ethanol. When cells were treated with 200 mM to 500 mM ethanol, the AChE activity in cells increased significantly and the induction was dose-dependent. Increased levels of AChE activity in apoptotic cells have been reported by other groups (Cai et al., 2013; Zhang and Greenberg, 2012). Our results confirmed that ethanol treatment induced cell apoptosis together with AChE expression, suggesting that AChE may be related to apoptosis of human SH-SY5Y Cells.

4.2. *Excess AChE in plasma is a biomarker of toxicity*

AChE has emerged as an important contributor to apoptosis in various types of cultured cells. However, whether the increased AChE levels are associated with ethanol-induced apoptosis in mice was unknown. In the present work, plasma carboxylesterase knockout mice (ES1 −/− BChE +/+) were used as models for humans because humans have no plasma carboxylesterase (Li et al., 2005). In a previous report, we used wild-type mice as well as double knockout mice ES1 −/− BChE +/− and ES1 −/− BChE −/− that were partially or completely deficient in BChE (Duysen and Lockridge, 2011; Jiang et al., 2012). All genotypes responded to OP treatment with induction of plasma AChE activity. This suggests that plasma carboxylesterase and butyrylcholinesterase do not play a role in induction of excess AChE activity.

It is established that ethanol induces apoptosis in mouse tissues (Jana et al., 2010). We treated mice with ethanol for the purpose of determining whether ethanol-stimulated apoptosis was associated with the appearance of excess AChE in plasma. Mice treated with ethanol had minimal behavioral signs of toxicity, yet they had abnormally high plasma AChE activity lasting for several days. Ethanol causes neurotoxicity, suggesting that neurons undergoing apoptosis may be one source of the excess AChE in plasma. Mice have significant amounts of AChE in plasma, while in healthy humans the concentration of AChE in plasma is minimal about 8 ng/ml (Brimijoin and Hammond, 1988). It was demonstrated that ethanol treated mice had more apoptotic cells in brain tissue than were found in untreated control mice, and apoptotic cells were associated with high levels of AChE activity. The increased AChE in plasma could be the consequence of apoptotic cells releasing AChE into the circulation. We conclude that excess plasma AChE activity is associated with apoptotic cells and is an indicator of apoptosis.

4.3. *Roles of AChE in neurodegenerative diseases*

In apoptotic cell death, AChE is necessary for apoptosome assembly and the activation of the initiator caspase-9 (Zhang and Greenberg, 2012). Du et al. (Du et al., 2015) showed that recombinant synaptic acetylcholinesterase (AChE-S) has endonuclease activity, indicating that AChE is a true apoptotic DNase. The nuclear redistribution of AChE-S correlates with TUNEL staining, which led us to speculate about the potential role of AChE-S in neurodegenerative disorders. Heavy alcohol use was reported to be associated with neurodegenerative diseases (Huang et al., 2016; Ridderinkhof et al., 2002; Weissenborn and Duka, 2003). AD patients have 20% higher AChE activity in plasma compared to age and gender-matched controls with no change in BChE levels (Garcia-Ayllon et al., 2010). Elevated levels of plasma AChE activity in AD imply that this enzyme may be playing a pivotal role in the pathogenesis of the disease (Mushtaq et al., 2014).

Acetylcholinesterase inhibitors could attenuate H_2O_2-induced apoptosis in human neuroblastoma SH-SY5Y cells (Yao et al., 2016). AChE inhibitors had multiple anti-apoptosis neuroprotective potentials (Kalb et al., 2013; Xie et al., 2016; Zhang et al., 2016) and showed a potential therapeutic value in the treatment of early AD (Carvajal and Inestrosa, 2011; Toiber et al., 2008). The AChE inhibitors donepezil and rivastigmine are approved by the US Food and Drug Administration (FDA) as a first-choice therapy for the treatment of mild to moderate Alzheimer's disease (Zemek et al., 2014). These AChE inhibitors showed beneficial effects on acute ethanol-induced cognitive impairments in rats (Gawel et al., 2016). The mechanism for ethanol-induced brain damage and the role of AChE are still not clear. Therefore, it should be of interest to explore the cytotoxic mechanisms to identify potential tools of therapeutic benefit (Sanchez-Osuna and Yuste, 2015). How AChE contributes to ethanol neurotoxicity warrants further study and might give some hints for therapeutic interest in the treatment of related diseases.

5. Conclusion

Cells exposed to ethanol had increased apoptosis and AChE activity. An overall increase in plasma AChE activity correlates with increased apoptotic cells in ethanol treated mice. Cells staining for AChE activity also stain for apoptosis in mouse brain. Taken together, these observations strongly suggest that increased AChE activity is a biomarker for apoptotic activity in both cells and mice exposed to ethanol. However, the exact role of AChE in ethanol-induced apoptosis remains unclear, warranting further study of the role of AChE in ethanol-induced neurotoxicity.

Conflicts of interest

The authors declare no conflict of interest.

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