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Introduction

Acetylcholinesterase (AChE; EC: 3.1.1.7) is an essential enzyme, which rapidly hydrolyzes the neurotransmitter, acetylcholine to acetate and choline. Liver is an important source of blood AChE (Garcia-Ayllon et al., 2006), and the alterations in the peripheral cholinergic system has been implicated in neurological diseases like Alzheimer’s disease (Rakonczay et al., 2005), progressive muscular atrophy (Rasool et al., 1983), liver failure (Rao et al., 1994; Kabatnik et al., 1999; Jamal et al., 2007; Swapna et al., 2007). AChE is also found in the motor ends plates of skeletal muscle, central nervous system and erythrocytes (Kutty, 1980). However, Butterworth and associates (Rao et al., 1994) failed to demonstrate changes in the levels of cholinergic enzymes in human and experimental portal systemic encephalopathy.

Hepatic encephalopathy (HE) is a complex neuropsychiatric syndrome that occurs as a consequence of acute or chronic hepatic failure (Chalasani and Gitlin, 1996; Jones and Weissenborn, 1997). Numerous injurious substances such as ammonia, alcohol, hepatitis-B/-C infection, pigment deposition, cholestasis etc. are suggested to participate in the progression of HE (Butterworth et al., 1987; Lockwood et al., 1991; Rose et al., 1999; Blei et al., 2001; Butterworth et al., 2003, Baumert et al., 2007, Sigal SH, 2006; Uchida et al., 1983). Different grading of encephalopathy (grade 0 to IV) occurs due to some agents /precipitating factors such as oral protein load, constipation, gastrointestinal bleeding, electrolytes imbalance, hypoglycemia, sedative / hypnotic drugs (Haggerty, 2002). The clinical facial appearance of HE include changes in mood and personality, sleep, cognitive, psychiatric conditions such as anxiety and depression, as well as motor disturbances (Jones and Weissenborn, 1997). These symptoms impair function and quality of life (Blei and Corboda, 2001). Our aim of the present study is to find out whole blood indicators that correlate with the severity of the HE and neurological deficits. In this Chapter we studied the activity of an important enzyme of the cholinergic system, acetylcholinesterase (AChE) in relation with HE.
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Plasma from bile duct ligation rats has been shown to have approximately 45% lower AChE activity than controls (Garcia-Ayllon et al., 2006). Recently a study (Garcia-Ayllon et al., 2008) on cirrhotic patients and bile duct ligation model in rat showed 30% and 20% increase of AChE activity, respectively. Several authors have previously described the distribution of cholinesterases in the liver of rat (Wheeler et al., 1972, Satler et al., 1974, Perelman and Bandan, 1989, Berninsone et al., 1989), mouse (Golmez et al., 2000), rabbit (Ballantyne, 1978, Jbilo et al., 1994), and chick (Smucker et al., 1990). Rat is a good animal model to study the effect of liver AChE levels, because other cholinergic enzymes such as butyryl cholinesterase activity in rat is much lower than in human blood (Garcia-Ayllon et al., 2006). We used thioacetamide (TAA) as an effective hepatoxin that has been shown to induce fulminant hepatic failure (FHF) in rat in a short period of time (Albrecht et al., 1990; Shapiro et al., 2006; Zimmermann et al., 1989; Larsen et al., 1994; Bruck et al., 1999). Our data supports that alteration of cholinergic neurotransmission may be involved in the pathogenesis of HE.

Results

Whole blood AChE activity increase with gradation of HE

HE patients showed a pronounced increase in whole blood AChE activity (5.68 ± 0.19 μmol/min/mg protein) when compared to the normal subjects and without hepatic encephalopathy (WHE) patients (3.45 ± 0.13 μmol/min/mg protein and 4.94 ± 0.22 μmol/min/mg protein; respectively, Fig. 1A). There was a significant increase of AChE activity in WHE as well as HE-0 to HE-IV groups of patients when compared with normal subjects. The AChE activity in HE-III and HE-IV is significantly increased than the WHE group (Fig. 1B). In the animal model, AChE activity was 1.8-fold increased in rats those received higher dose of TAA (200 mg/kg/day) as compared to the control group. Rats receiving 100 mg/kg/day dose showed only 1.3 fold increase (Fig. 1C).
Fig. 1. Whole blood acetylcholinesterase (AChE) activity in patients (A, B) and TAA-treated rat (C). The activity of AChE was measured by the method of Ellman et al. (1961). Specific AChE activity is expressed as μmol of 5-thio-2-nitrobenzoic acid formed per minute per milligram protein. A depicts whole blood activity of AChE in normal individuals, in patients with and without encephalopathy. B whole blood activity of AChE in normal, in patients WHE and the different gradation of HE. C whole blood activity of AChE in control, 100 & 200 mg/kg TAA treated rats. Results are given as Mean ± S.E.M. (N-Normal; n = 50 humans; 10 rat); *p < 0.001 as compared among HE & WHE and normal subjects. * compared between WHE and HE, or between two TAA doses.

Different gradations of HE and whole blood acetyl cholinesterase (AChE) activity

Correlation data showed that whole blood AChE activity increases from ‘0’ grade to grade ‘IV’ subjects (r = 0.685, Fig. 2A) and the increase is highly significant. A positive correlation was also found between the AChE activity with the doses of TAA-treated in FHE rats (r = 0.537, Fig. 2B).

Fig. 2. Relationship of the different gradations of HE (A) and TAA-treated rat (B) with that of whole blood acetyl cholinesterase (AChE) activity. A provides correlation scatter plot for cirrhotic patients’ whole blood activity for AChE in relation to them exhibiting 0-IV grades of encephalopathy (N - Normal; n = 100; r = 0.685). B is the correlation plot for AChE activity in rat whole blood in relation to TAA doses (n = 30; r = 0.537).
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AChE activities in regions of rat brain

We measured AChE levels in the nucleus caudatus putamen (NCP), cortex (CR), Hippocampus(HP), and nucleus raphe dorsale (NRD) because these brain regions are important for cognition, learning and memory formation and in neurodegenerative diseases cholinergic nerve endings within these regions are typically affected (Dutar et al. 1995). The first important finding of this study was the increased activity of AChE in the CR and HP of TAA treated rats in both the doses. The estimated activity with the higher dose in CR and HP are 13.30 ± 1.53, and 41.71 ± 1.89 μmol/min/mg protein; respectively. With the lower dose the activities are 9.55 ± 0.53, and 32.43 ± 1.45 μmol/min/mg protein. Other regions were unaffected.

Fig. 3. Effect of TAA treatment on AChE activity in discrete rat brain regions. AChE activity (μmol/min/mg protein) was assayed in four regions of brain employing Ellman et al., (1961), procedure. Results are given in Mean ± S.E.M. (NCP = nucleus caudate putamen, CR = cortex, HP = hippocampus, NRD = nucleus raphe dorsale; n = 6); *p < 0.05 as compared to control and @ p < 0.05 as compared to lower dose (100 mg/kg).

Analysis of whole blood AChE in normal subjects and patients (with or without HE) by in gel activity

Gels revealed one enzymatically active acetylcholinesterase band in the blood suspensions after staining. As expected for the acetylcholinesterase staining, the HE patients' blood suspension showed a single band (Fig. 4A). Densitometric analyses showed an average 1.57-fold increase in peak intensity in HE (215569.3 ± 8272.63 INT/mm²) and 1.21- folds in WHE (166482.5 ± 10537.67 INT/mm²) when compared to that of the normal subjects (137170 ± 8226.74 INT/mm²) (Fig. 4B).
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Fig. 4. Polyacrylamide gel electrophoresis with 0.5% Triton X-100 for AChE staining.
5 μg/μl protein in each lane was loaded along with an AChE standard. After run gel was transferred onto acetylthiocholine (pH 6) contains staining solution and incubated at RT, until the bands appeared on the gel. A show that the activity wise WHE and HE samples are showing very intense bands. B is a representative of the peak intensities. Results are given as Mean ± S.E.M. (n = 3); *p< 0.001. *compared between HE and normal subjects.

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**AChE protein expression levels**

For western blotting of AChE protein, we employed a monoclonal antibody raised towards the protein. AChE protein band was detected at approximately 75 KD in the blood suspension of normal, WHE and HE patients (Fig. 5A). Beta actin was taken as the loading control. The intensities of the bands were noted and were divided by the loading control for getting the normalized values (Fig. 5B). This result confirms that there is enrichment of whole blood AChE proteins in the HE patients.

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Fig. 5. AChE protein expression levels. The protein level of AChE from normal volunteers, WHE & HE patients was measured by western blotting. 2.5 μg/μl proteins each from the normal volunteers, WHE, and HE patients were loaded in 10% SDS-polyacrylamide gel, separated by electrophoresis, transferred onto PVDF membrane and probed with mouse anti-acetylcholinesterase antibody. After developing with DAB, blot showed a band of 75 KD for AChE. A is representative from three immunoblot analysis. B represents AChE band intensities of 75 KD for HE patients. Results are given as Mean ± S.E.M (n = 3); *p≤ 0.001. *compared between HE and normal subjects.
Brain morphology

Stereomicroscopic images of sections passing through the NCP and cortex when stained for AChE activity showed no overt pathology (Fig. 6).

Fig. 6. AChE activity in coronal sections of rat brain. Saline or TAA (200 mg/kg) were injected intraperitoneally once daily for three days and the animals were perfused with 4% PFA on the 4th day. The fixed brains were cryo-cut coronally (20 μm sections). Representative photographs of coronal sections passing through NCP from control (A), and treated rats (B) showed high level of AChE activity in NCP, and low activity in the CC, SR, and MS regions (magnification 12 x). (C) & (D) represent sections passing through the SGC and PAG (magnification 32 x). NCP = nucleus caudatus putamen, CC = cerebral cortex, SR = septal region, MS = medial septal nucleus, SGC = substantia griesea centralis, PAG = peri aquaeductal grey.

Discussion

AChE activity has been found to be significantly higher in the blood samples of HE patients than the ones without HE (Fig. 1A). It was found that there is a positive correlation between blood AChE activity and severity of HE in patients (Fig. 2A). It was also reflected in our animal model where the AChE activity showed dose dependent increase (Fig. 1B). The AChE activity in HE-II, HE-III and HE-IV significantly increases by 2.46, 3.9 and 4.64- folds, respectively when compared to the normal subjects (Fig. 2C). From this study we can conclude that AChE levels WHE, HE-I and HE-II can be well distinguished from HE-III and HE-IV. Hepatocyte is rich in AChE. This enzyme gets released from there into the blood which distributes it to its target sites (Garcia-Ayllon et al., 2006; Perelman and Bandan, 1989). When the hepatocytes are injured due to some liver damaging factors, AChE may be appear into the blood that is why we are getting increased activity. AChE may also be involved in some intercellular and intracellular regulatory mechanisms (Satler et al., 1974). Richard et al. (2005) described that the activities of AChE in red blood cells and butyrylcholinesterase in serum can be used as potential biomarkers of suppressed and / or heightened activity in...
the central and peripheral nervous systems. We observed that a significantly higher AChE activity in higher dose (200 mg/kg) of TAA administered rat blood samples of FHF, as compared to the blood taken from the lower dose (100 mg/kg) administered animals (Fig. 2B).

In different rat brain areas, such as cortex and hippocampus, AChE activity was significantly increased (Fig. 3), which is in accordance to the increase in the whole blood. Hippocampus showed a four-fold increase of AChE activity, coupled which strongly suggests the presence of severe mentation. Liapi et al. (2009) observed similar results that the rat brain AChE was significantly increased by TAA administration. In agreement with the human data, AChE activity in the brain cortical extracts of bile duct ligated rats was increased (~20%) compared to controls and in the brain frontal cortex extracts of HE patients was increased (33%) compared to non-cirrhotic controls (Garcia-Ayllon et al., 2008). Interestingly, significant AChE increase after acute ethanol exposure has been demonstrated in the zebrafish brain, whereas ethanol in vitro did not alter enzymatic activity (Rico et al., 2007). Garcia-Ayllon et al. (2008) showed that 30% and 20% increases of levels of cholinergic enzymes (AChE) in the brain of cirrhotic patients and bile duct ligated rats. Recently, Mendez et al. (2010) reported altered AChE in the entorhinal cortex, anterodorsal and anteroventral thalamus and accumbens in Wistar rats treated with thioacetamide. We studied AChE activity using polyacrylamide gel electrophoresis (PAGE) in patients and normal healthy subjects (Fig. 4A) and observed one single band when stained specifically for AChE staining and a significant increase in peak intensities (~16%) in whole blood of the HE patients (*p ≤ 0.05, Fig. 4B). Furthermore, to identify that the homogenized whole blood was enriched with the protein, we performed western blot analysis with the specific antibody (monoclonal mouse anti-AChE) for whole blood HE patients’ proteins. Accordingly, we detected a protein band of approximately 75 kD, (Fig. 5A), which was significantly increased (Fig. 5B). We concluded from these findings that AChE level in human whole blood may be an efficient way to detect the disease.
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Conclusion

A significant positive correlation among blood AChE activity and severity of HE in patients, and thioacetamide-treated rats strongly suggests that this enzyme activity is a possible peripheral marker for the presence and absence of encephalopathy in patients and rats. This is the first proof of a cholinergic imbalance in the brain as well as in the whole blood as an effect of liver failure and points to the probable role of the cholinergic system in pathogenesis of peripheral hepatic encephalopathy.

References


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