Abstract. – Objectives: Metals such as iron are needed for physiological functions of the body. However, their excess may cause serious damage including poisoning. The effects of Fe²⁺ and Fe³⁺ ions on human plasma cholinesterase activity were investigated.

Material and Methods: Plasma were obtained (n=24) from healthy male volunteers, and treated with five various concentrations of Fe²⁺ and Fe³⁺ ions. Activity of butyryl cholinesterase (BChE) was assayed according to colorimetric Ellman procedure. The activity was calculated as micromole of hydrolyzed substrate, per minute in each ml.

Results: Both ferric (Fe³⁺) and ferrous (Fe²⁺) ions significantly decreased the BChE activity in all tested concentrations. The effect of ferric (Fe³⁺) ions on the reduction of plasma enzyme activity was more pronounced than ferrous (Fe²⁺) ions (p<0.001, respect to control group).

Conclusions: The present paper represents the effect of Fe²⁺ and Fe³⁺ ions on ButyrylChE activity in human plasma. It reveals that both ions suppress BChE activity.

Key Words: Ellman procedure, Iron, Fe²⁺ ions, Fe³⁺ ions, Plasma cholinesterase, BChE activity.

Introduction

Heavy metals, pesticides, chemical sewage and waste water can effect environment. Since these compounds inhibit cholinesterase (ChE) activity, determination of activity of this enzyme is a reliable and widely used biological indicator for exposure to these agents. There are two different types of ChE in human body, which differ in their location in tissues, substrate affinity, physiological function and sensitivity toward various inhibitors: acetyl cholinesterase (AChE; E.C. 3.1.1.7) which mainly found in the brain, muscles, erythrocytes and cholinergic neurons. ChE plays a major role in the regulation of several physiological events by hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses. Butyryl cholinesterase (BChE; E.C. 3.1.1.8) is present in glial cells, plasma, originates mainly from the liver and is capable of splitting several choline esters, including acetylcholine. This group of enzymes is present not only in liver but also in intestine, kidney, heart, lung, serum and cholinergic synapses, within CNS and plays a major role in the metabolism of ester containing compounds. BChE can also take the place of AChE in the acetylcholine degradation, when acetyl cholinesterase is inhibited or absent. Therefore, the knowledge of the cholinesterase status is crucial for the early diagnosis of organophosphate pesticides (OPs) exposure or intoxication and for monitoring the therapeutic effects of reactivates. The inhibiting power on AChE and BChE varies widely among the different OP compounds. Some OPs inhibit AChE more strongly and some others BChE. Some others are stronger inhibitors of BChE than AChE. In exposure to these substances, plasma BChE determination is a more sensitive indicator of exposure than AChE. However, BChE inhibition is not associated with symptoms or signs of toxicity.

Among the bivalent metal ions, the effects of Hg²⁺, Cd²⁺, Cu²⁺, Mg²⁺ and Ca²⁺ on BChE from different sources have been investigated. Al³⁺, also has been showed to inhibit sheep brain BChE. Plasma cholinesterase can be depressed by inherited traits or by other causes, notably liver disease. Thus, estimation of AChE activity gives us more reliable information in these situations. Iron ions (Fe²⁺ and Fe³⁺) have many functions in body including oxygen transport and enzyme activity. The main function of iron is to...
help make hemoglobin which attracts oxygen from the air one breath\(^1\). Recently we have reported the effect of iron ions (Fe\(^{2+}\) and Fe\(^{3+}\)) on AChE activity\(^2\). However, the effect of iron ions on the human plasma cholinesterase activity is unclear. The purpose of this investigation was to evaluate the effect of ferric and ferrous ion on human BChE activity, in continuation of previous our researches on the determination of AChE activity in human and fishes\(^3,8,9\).

**Materials and Methods**

**Chemicals**

Butyrylthiocholine iodide (BTCh), dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (St. Louis, MO, USA). FeSO\(_4\), Fe\(_2\)(SO\(_4\))\(_3\), and other compounds used in this study were prepared from Merck Chemical Co. (Darmstadt, Germany). Water was distilled and deionized.

**DTNB Reagent**

It was prepared in 1 liter of 33 mmol/L (5.74 g/L) K\(_2\)HPO\(_4\) and 100 ml of 0.10 mol/L KH\(_2\)PO\(_4\) (1.36 g/100 ml). Enough amounts of the latter were added to bring the pH of the former to 7.6. Then, 0.27 mmol (107 mg) of DTNB was prepared in 1 L of the buffer and stored in a dark bottle at 4°C.

**BTCh Substrate**

634 mg of BTCh was dissolved in 10 ml of water (0.2 mol/L). Immediately before the use, the substrate was diluted with 1.0 ml of water and mixed thoroughly.

**Stopping Reagent**

This was 43 mmol/L (20 g/L) of hyamine 1622 in water, which was stored at 4°C in a dark bottle.

**Ferric Chloride Solution**

Ferric treatment was done by adding certain concentration of ferric stock solution (290.229 mg/L of FeCl\(_3\), 6H\(_2\)O) to prepare 50, 75, 100, 200, 250 µg/dl ferric per plasma.

**Ferrous Sulfate Solution**

Ferrous treatment was done by adding certain concentration of ferrous stock solution (298.519 mg/L of FeSO\(_4\), 7H\(_2\)O) to prepare 50, 75, 100, 200, 250 µg/dl ferrous per plasma.

**Samples**

Blood samples were obtained from 24 healthy volunteers (ages 28 to 35) who were not exposed to ferrous or ferric ion and any ChE inhibitors. Heparinized blood samples were centrifuged at 6,000 rpm, to separate erythrocytes from plasma. 100 µL of the supernatant layer was used. Activity was assayed by method described below.

**Estimation of Activity**

The estimation of BChE activity was performed according to the method of Ellman et al\(^10\) which has been modified by George and Abernethy\(^11\). The enzyme activity was calculated by measuring the increase in yellow color produced by thiocholine when it reacts with dithiobis nitrobenzoate ions. A double beam spectrophotometer (UV – Visible EZ201, Perkin Elmer: Norwalk, CA, USA) was used for estimating enzyme activity. The analyses were performed at 25 ± 1°C at wavelength of 440 nm.

**Series A:** To 6 glass test tubes containing 50 µl of plasma, several concentration levels of ferric chloride stock solution or ferrous sulfate stock solution was added to prepare solutions of 50, 75, 100, 200, 250 µg/dl of ferric or ferrous per ml of plasma. These solutions were incubated for 30 minutes in a water bath set at 37°C and then were used for enzyme activity assay. Blank tube was 50 µl of plasma, without ferric or ferrous addition.

**Series B:** To 7 glass test tubes containing 3 ml of DTNB reagent and 100 µl of BTCh substrate was equilibrated. After 25 minute intervals, the series A were added to start a reaction. Tubes were placed in the water bath set at 37°C. To 6 glass test tubes of B series, 100 µL of a series samples was added. To number 7 glass test tubes of B series as controls and without further addition (metals ion or plasma) 100 µl of distilled water was added. Exactly 10 minutes after mixing, 1.0 ml of Hyamine 1622 stopping reagent was added. The tubes were placed for 30 minutes at room temperature. Absorbance of test solution vs. the respective blanks was measured at 440 nm. For calculation of the resulting plasma cholinesterase activity (R) in µM Bch/hydrolyzed/min unit (U) per ml of plasma, absorbance difference (\(\Delta A\)) was multiplied by 1/C (primary substrate concentration) and reaction time was 10 minutes i.e R = 574 \(\Delta A/C\) . Triplicate measurements were done for each sample, and the mean value was taken.
Analysis of the Data

Statistical analysis was performed using SPSS for Windows (Ver. 10, SPSS, Inc., Chicago, IL, USA). Data was analyzed by one-way analysis of variance (ANOVA) and presented as means ± standard deviation (SD). Student-Newman-Keuls test was used for statistical analysis and \( P<0.05 \) was considered to be significant.

Results

Figure 1 shows correlation between the mean concentration (Means ± SD) of ferric (Fe\(^{3+}\)) and ferrous (Fe\(^{2+}\)) ions per dL of plasma vs BChE activity of samples from healthy volunteers. There was a significant difference between mean BChE activity in Fe\(^{2+}\) and Fe\(^{3+}\) treated samples. This activity in Fe\(^{3+}\)-treated samples was significantly lower than the activity in Fe\(^{2+}\)-treated ones (\( P<0.05 \)). The effect was concentration dependent, but the activity was lowest at 75 \( \mu \)g/dl for Fe\(^{3+}\) and at 200 \( \mu \)g/dl for Fe\(^{2+}\). As shown in Figure 1, both ferric (Fe\(^{3+}\)) and ferrous (Fe\(^{2+}\)) treatments significantly (\( p<0.01 \) or \( p<0.001 \)) decreased the BChE activity in all tested concentrations. The effect of ferric (Fe\(^{3+}\)) ions on the reduction of plasma enzyme activity was more pronounced than ferrous (Fe\(^{2+}\)) ions.

Discussion

Despite BChE abundant presence in different tissues, the physiological function of this enzyme has not yet been established. A variety of methods (electrometry, tintometry, radiometry and colorimetry) have been developed, providing sensitive and specific assay for determination of ChE activity. However, routine use of these procedures is often hampered by many factors such as, laborious sample preparation, long measuring time, insufficient specificity of substrate and disturbances by the sample matrix. The colorimetric Ellman et al procedure, is generally preferred method screening and therapeutic monitoring of these enzymes. The activity of ChE is reported to vary in different organs in response to environmental stress, including heavy metal stress.

Interest in the interaction of the ChE with metal ions has grown in recent years. Among the bivalent metal ions, the effects of Hg\(^{2+}\), Cd\(^{2+}\), Cu\(^{2+}\), Mg\(^{2+}\) and Ca\(^{2+}\) on BChE from different sources have been investigated. For brain BChE purified from sheep, it has been shown that Cd\(^{2+}\) and Zn\(^{2+}\) are hyperbolic mixed-type inhibitors of the enzyme and Ca\(^{2+}\) or Mg\(^{2+}\) reactivates the enzyme after Cd\(^{2+}\) or Zn\(^{2+}\) inhibition. Al\(^{3+}\) also inhibits human serum cholinesterase. Inhibition with Cd\(^{2+}\) or Zn\(^{2+}\) showed hyperbolic mixed-type inhibitors, but Al\(^{3+}\) was a linear mixed-type in-
hibitor of the sheep brain BChE\textsuperscript{22}. Cd\textsuperscript{2+}, Zn\textsuperscript{2+} and Al\textsuperscript{3+} are the linear mixed-type inhibitors of BChE in human and their kinetic parameters have been reported recently\textsuperscript{15}. As determined in present study, the human plasmas cholinesterase were exposed to five different concentrations of ferric (Fe\textsuperscript{3+}) or ferrous (Fe\textsuperscript{2+}) ions. They exhibited a remarkable decrease in BChE activity. The decrease in BChE activity could be due to the binding of Fe\textsuperscript{3+} or Fe\textsuperscript{2+} ions to lipid rich structural components of mitochondria and subsequently impairment of the activities of enzymes associated directly with lipid rich fractions, especially where the integrity of the structural components is necessary for the maximum catalytic activity. The activity of cholinesterase is organ-dependent and is attributed to the organs. Additionally, the magnitude of metal accumulation in the target tissues and biological half-life of the metal are considered the prime importance in the modulation or modification of the ChE\textsuperscript{23}.

Our results show significant difference between the rate of BChE inhibition by ferric (Fe\textsuperscript{3+}) and ferrous (Fe\textsuperscript{2+}) ions. As shown in Figure 1, both (Fe\textsuperscript{2+}) and (Fe\textsuperscript{3+}) ions demonstrate deep BChE inhibition. Ultimately, in vitro depression of plasma BChE activity can be caused by the direct effect of metal ions, i.e. a decrease in quantity of the enzyme, or it may be due to the interaction of metals and sulfhydryl groups of the enzyme. Cholinesterase binds its substrate by dipole/ionic interactions between charged and polar groups on the molecule. During the catalytic cycle, part of the substrate is also bound covalently. Co-factors of metal ions, such as (Fe\textsuperscript{3+}) and (Fe\textsuperscript{2+}) ions, are often bound by dipole interactions with histidine and other amino acids, including lone-pairs in catalase\textsuperscript{24}. Thus in vitro depression of plasma BChE activity can be caused by indirect effect of metal ions, when the ratio of ferrous (Fe\textsuperscript{2+}) to ferric (Fe\textsuperscript{3+}) drops below 8:1. The ideal situation for the development of the hydroxyl radical exists. In the presence of H\textsubscript{2}O\textsubscript{2}, ferrous, iron (Fe\textsuperscript{2+}) becomes oxidized to form ferric iron (Fe\textsuperscript{3+}), because it has lost an electron. The H\textsubscript{2}O\textsubscript{2} gains an electron and becomes reduced by forming the hydroxyl (OH) radical, which is a potential cause of lipid peroxidation\textsuperscript{6}.

The present paper reveals that both Fe\textsuperscript{3+} and Fe\textsuperscript{2+} ions are an essential metal or ions which suppress BChE activity in human plasma. The direct interaction of metal ions with BChE is proposed as a mechanism for depressed enzyme activity.

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