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AGE-DEPENDENT DECLINE IN ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY: CORRELATION WITH OXIDATIVE STRESS

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Background: Oxidative stress hypothesis offers a mechanism for the aging process and its involvement in other pathologies such as diabetes and neurodegenerative diseases like Alzheimer. AChE activity in erythrocytes may be considered as a marker of central cholinergic status. The present study was undertaken to (i) determine the activity of erythrocyte AChE as a function of human process (ii) correlate AChE activity with oxidative stress during human aging.

Material and Methods: Blood was collected from healthy subjects (n = 37) 22–82 years. Erythrocyte AChE activity, MDA and plasma antioxidant capacity in terms of FRAP was measured spectrophotometrically.

Results: There was a marked decrease in AChE activity with increasing age. The reduction in activity of AChE correlated well with increased lipid peroxidation and a decrease in FRAP values.

Conclusion: Decreased antioxidant defense, and alteration in membrane rheology during aging process both may contribute towards decreased activity of AChE in erythrocyte membrane. This finding may help in explaining the neuronal complications taking place under conditions of oxidative stress, aging, and dementia.

INTRODUCTION

Aging is an inevitable biological process and has been defined as the progressive accumulation of diverse deleterious changes with time that increases the chance of disease and death. The oxidative stress hypothesis offers a possible mechanistic explanation of aging process and plays an important role in Alzheimer's[1] and other neurodegenerative diseases[2]. In human aging and dementia, multiple neurotransmitter systems appear to be compromised[3, 4]. An age-related decline in cholinergic function is thought to be partially responsible for short-term memory disorders during senescence. The major marker of cholinergic metabolism is the activity of the hydrolytic enzyme acetylcholinesterase (AChE) that makes possible precise temporal control of synaptic activation by rapidly hydrolyzing neurotransmitter acetylcholine (ACh) into acetate and choline[5].

It is known that the activity of AChE decreases with aging in various cerebral areas[6] and synaptic plasma membranes[7]. Inhibition of AChE results in severe cholinergic toxic signs caused by increased concentration of acetylcholine at cholinergic nerve-nerve and nerve-muscle synapses. Despite the fact that, the biological role of the acetylcholinesterase present in the erythrocyte membrane is unknown, this enzyme is reported to have many properties similar to those of the purified form obtained from brain tissues[8]. AChE activity in erythrocytes may be considered as a marker of central cholinergic status[9].

Many *in vitro* studies have indicated that several parameters of blood are negatively affected by increased oxidative stress and aging. It is also known that AChE activity is inhibited by free radicals and increased oxidative stress[10]. We have recently reported an age dependent increase in lipid peroxidation index MDA and a decline in the total antioxidant status of plasma[11, 12]. The aim of the present study was to investigate the effect of human aging on erythrocyte membrane-bound AChE and determine the correlation between markers of oxidative stress namely: lipid peroxidation and antioxidant status of plasma.

MATERIALS AND METHODS

The study was carried out on 37 normal healthy subjects of both sexes between 22 and 82 years of age. None of the subjects studied had been taking antioxidant supplementation and none smoked or had acute or chronic diseases or were receiving prescription medication. All individuals were healthy, without arterial hypertension, diabetes mellitus, asthma, cancer or tuberculosis and were well nourished. The subjects gave their informed consent and agreed to participate in the study and donation of blood samples. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

Human venous blood from healthy volunteers was collected in heparin tubes . Blood samples were centrifuged at 4 °C for 10 minutes at 100 g to remove plasma and buffy coat and isolated erythrocytes were washed four to five times with 0.154 mol/l NaCl.

Determination of AChE activity Erythrocyte hemolysate was prepared as described by Beutler[13]. Packed RBC were suspended in 0.154 M NaCl and to this suspension, β-mercaptoethanol-EDTA stabilizing solution was added

and the hemolysate was frozen overnight. The hemolysate was thawed preceding the experimental procedure. The membrane bound acetylcholinesterase activity in the human red blood cell was analyzed following the method of Ellman et al.[14] as described in Beutler[13]. The reaction mixture composed of 1mol/l Tris HCl, 5mmol/l EDTA with 0.5mmol/l 5,5′–di-thiobis (2-nitro-benzoic acid) (DTNB) solution. The reaction was initiated by adding 0.01 mol/l acetylthiocholine iodide and followed by reading at 412 nm. Hemoglobin was measured in red blood cell hemolysate as described by Beutler[13].

Determination of MDA Content Erythrocyte MDA was measured according to the method of Esterbauer and Cheeseman[15]. Packed erythrocytes (0.2 ml) were suspended in 3 ml Krebs– Ringer phosphate buffer (KRP), pH 7.4. The lysate (1 ml) was added to 1 mL of 10 % trichloroacetic acid (TCA) and the mixture was centrifuged at 1000 *g* for 5 min. The supernatant (1 ml) was added to 1ml of 0.67 % thiobarbituric acid (TBA) in 0.05 mol/l NaOH and boiled for 20 min at 90°C, cooled and the absorbance was read at 532 nm (OD1) and 600 nm (OD2). The net optical density (OD) was calculated after subtracting absorbance OD2 from OD1. The concentration of MDA in erythrocytes was determined from a standard plot. The concentration of MDA was expressed as nmol/ml of packed erythrocytes.

Determination of total antioxidant capacity The total antioxidant capacity was measured in terms of Ferric Reducing Ability of Plasma (FRAP) by the method of Benzie and Strain[16]. Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2, 4, 6- tri [2-pyridyl]-s-triazine (10 mM in 40 mM HCl) solution and $FeCl_3.6H_2O$ (20 mmol/l) solution in 10:1:1 ratio respectively. 3 ml of FRAP reagent was mixed with 100 µl of plasma and the contents were mixed thoroughly. The absorbance was read at 593 nm at 30 second intervals for 4 minutes. Aqueous solution of known Fe(II) concentration in the range of 100-1000 µmol/l was used for calibration. Regression equation the FRAP values (µmol Fe (II) per l) of the plasma was used for calculation.

Statistical analyses were performed using the software PRISM 4 (Graph pad Software Inc., San Diego, CA). The relationship among the various parameters was assessed using Pearson correlation coefficient (*r*).

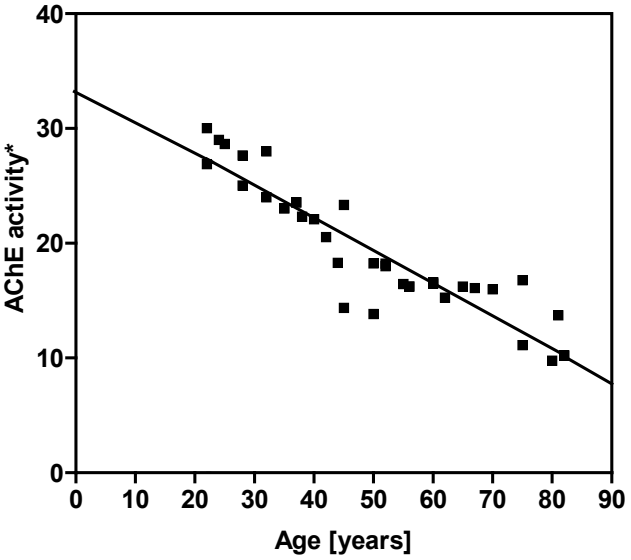

Fig. 1. Erythrocyte membrane bound acetylcholinesterase activity plotted as a function of human age. *Activity expressed as µmol acetylcholine iodide hydrolysed/min per gm haemoglobin at 37 °C. $P < 0.001$; $r = -0.9218$. Each point represents the mean of 3 experimental values.

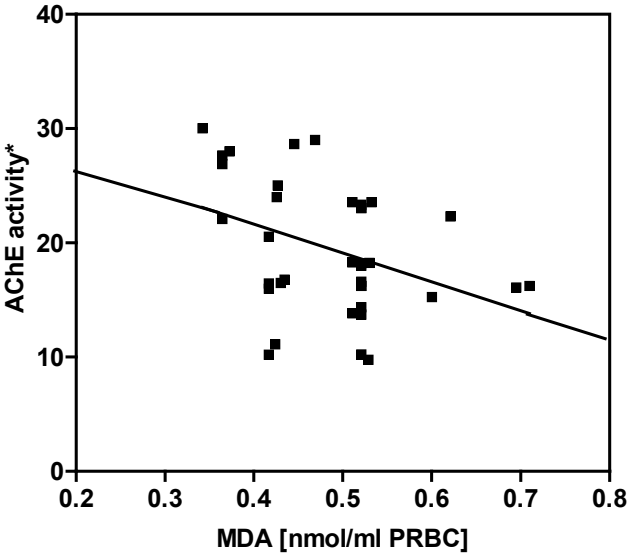

Fig. 2. Correlation plot between AChE activity and lipid peroxidation index (MDA). AChE activity expressed as µmol acetylcholine iodide hydrolysed/min per gm haemoglobin at 37 °C. MDA is expressed as nmol/ml of packed erythrocytes. $P < 0.05$; $r = -0.3791$.

RESULTS AND DISCUSSION

Human erythrocytes undergo extensive structural, chemical, and metabolic changes during aging. Our results show a significant negative correlation ($P < 0.001$; $r = -0.9218$) between erythrocyte membrane acetylcholinesterase activity and human age (Fig. 1). The study clearly indicates that the erythrocyte AChE activity decreases with increase in age of human subjects. The results are consistent with previous reports examining the relationship between aging and AChE activity in different brain regions[17, 18]. Decline in cholinergic indices (choline acetyltransferase, AChE, and muscarinic acetylcholine receptors) has already been reported during normal aging process[19].

Aging is the most important risk factor for Alzheimer's disease. Changes in cholinergic function have been characterized and a strong correlation has been observed with cognitive decline associated with aging. A low activity of AChE or cholinesterase present in cerebrospinal fluid of

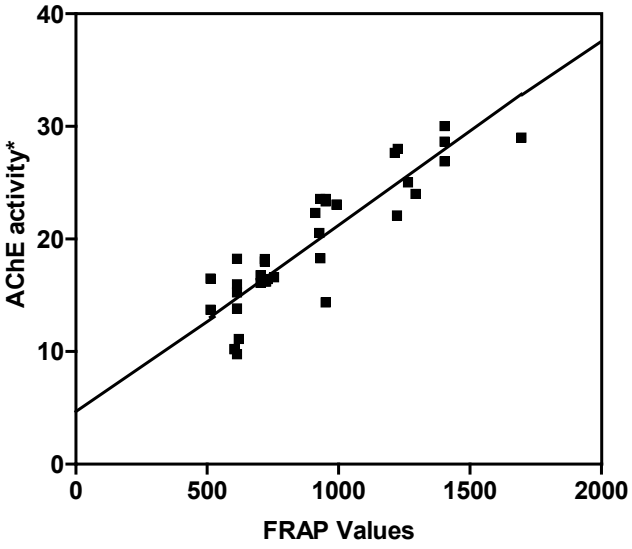

Fig. 3. Correlation plot between AChE activity and total antioxidant capacity of plasma (measured as FRAP). AChE activity expressed as µmol acetylcholine iodide hydrolysed/min per gm haemoglobin at 37 °C. FRAP values expressed as µmol Fe (II) per l of plasma. $P < 0.001$; $r = -0.8837$.

a non-demented individual may indicate a brain at risk, or that the person is in the preclinical stage of dementia[20].

The erythrocyte membrane is direct target of lipid peroxidation under oxidative stress that involves cleavage of polyunsaturated fatty acids at their double bonds leading to the formation of MDA. As lipid peroxidation of cell membranes increases, the polarity of lipid-phase surface charge and the formation of protein oligomers increase, and molecular mobility of lipids, number of -SH groups, and resistance to thermo denaturation decrease. Recent findings have emphasized the importance of lipid peroxidation in relation to the role of caloric restriction and the extension of longevity[21]. Fig. 2 shows the correlation between erythrocyte AChE and lipid peroxidation, measured in terms of MDA. We have already reported an age-dependent increase in MDA[11]. The decrease in AChE correlates significantly with increase in lipid peroxidation during human aging.

The activity of AChE depends largely on the membrane characteristics. It is known that AChE activity is modulated by the hydrophobic environment of the membrane and depends on the membrane fluidity and surface charge[22]**.** Membrane fluidity, which is a key property of the membrane lipid bilayer, has been found to decrease with aging[23]. These changes may cause alterations in the physical properties of membranes resulting in modifications in enzymatic activity of membrane bound proteins and lipid-protein interactions. It has also been reported that AChE activity is influenced by membrane surface phenomena[24]. A decrease in the erythrocyte membrane sialic acid content has been reported during aging[25], since sialic acid constitutes the principal charged component of the membrane, its loss from the erythrocyte surface during aging may be expected to reduce the total negative charge on the surface of the erythrocytes, and consequently their membrane potential. Furthermore, it has been suggested that the insertion of membrane proteins into the membrane lipid core may be dependent on transmembrane potential[26].

The correlation between antioxidant capacity and oxidative damage during aging has been reported in several tissues in different species[27-29]. Fig. 3 shows the correlation between erythrocyte AChE and total plasma antioxidant capacity, measured in terms of FRAP values. We have already reported significant age-dependent decline in plasma antioxidant capacity measured in terms of FRAP[12]. The decrease in AChE correlates significantly with decrease in the antioxidant capacity of the plasma during human aging.

A study carried out on red cells subfractionated on percoll density gradient on the basis of cellular age into old and young cell fractions, reported that old human red blood cells had significantly lower AChE activity compared to young human red blood cells of both the sexes. Based on this observation, it was suggested that AChE activity could be an excellent enzymatic marker for RBC aging in humans[5] although the plasma activity of AChE has been shown to be unaffected by age in humans[30]. Our results show significant age-dependent decrease in the activity of membrane bound AChE in a mixed a population of red blood cells and is the first report of an age-dependent decline in AChE in humans.

CONCLUSION

The decline in AChE activity with increasing human age seems to be closely correlated with increasing extracellular oxidative stress; since we also show the correlation of age related alteration in AChE with age dependent decline with total antioxidant capacity of plasma and with increased lipid peroxidation during human aging. Both decreased antioxidant defence along with alteration in membrane rheology during aging may contribute towards decreased activity of AChE activity in erythrocyte membrane. In the light of a number of reports emphasizing the contribution of cholinergic decline towards memory deficits observed in aging and Alzheimer's disease, our findings may help to explain the neuronal complications taking place under conditions of oxidative stress and aging, as well as in the development of dementia.

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