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Erythrocyte membrane acetylcholinesterase in type 1 (insulin-dependent) diabetes mellitus

Mohammad SUHAIL and Syed I. RIZVI
Department of Biochemistry, University of Allahabad, Allahabad-211002, U.P., India

1. The erythrocyte membrane acetylcholinesterase activity is significantly ($P < 0.001$) decreased in insulin-dependent diabetes mellitus. 2. The activity is negatively correlated ($r = -0.97$) with the fasting blood glucose level. 3. Insulin treatment restores the activity to normal. 4. The K_m of the enzyme for acetylthiocholine iodide was unchanged; however, the $V_{max.}$ was decreased, suggesting a decrease in the number of active enzyme molecules in diabetes.

INTRODUCTION

An earlier report from our laboratory has demonstrated that insulin plays an important role in the maintenance of erythrocyte membrane Na^+,K^+-ATPase microenvironment in human diabetics [1]. Insulin has been shown to stimulate acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) in slices of rat brain [2]. Despite the fact that, to date, the biological role of the acetylcholinesterase present in the erythrocyte membrane is unknown, this enzyme is being studied in the erythrocyte and is reported to have many properties similar to those of the purified form obtained from brain tissues [3]. Secondary complications in diabetes include neuropathy, which is characterized by a variety of morphological changes associated with decreased sensory and motor conduction velocities [4,5]; this alteration has been found to be corrected by insulin treatment [4,6]. A 32% reduction in the rate of acetylcholinesterase transport in cholinergic neurons has been observed in diabetic rats [7]. The present study on erythrocyte membrane acetylcholinesterase in type 1 (insulin-dependent) diabetes was undertaken to consider several alterations which take place in the diabetic erythrocyte membrane [8–10] and also to understand the effect of diabetes on membrane-bound acetylcholinesterase.

MATERIALS AND METHODS

Chemicals

All reagents were of highest purity available. 5,5′-Dithiobis-(2-nitrobenzoic acid) and Tris/HCl (Trizma hydrochloride) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Acetylthiocholine iodide was procured from Fluka AG, Buchs, Switzerland.

Selection of patients and blood collection

Patients with type 1 diabetes ($n = 19$; 12 male and 7 female) selected for the study were under varying degrees of diabetic control through insulin treatment, having their fasting blood glucose levels in the range 7.5–16.6 mM. The duration of diabetes had been 3–28 years. Healthy volunteers ($n = 15$; 11 male and 4 female) were taken as control subjects; none of the controls had any family history of diabetes mellitus. The fasting glucose levels of the controls ranged from 3.9–4.7 mM. Another group of type 1 diabetic patients ($n = 5$; 3 male and 2 female) selected for the study were under total control by insulin, with fasting blood glucose levels of 4.6–5.5 mM; these patients were termed insulin-treated diabetics. All the patients selected were non-obese and were screened for any clinical evidence of pancreatitis, and subjects having haemoglobin levels of less than 14 g/100 ml of blood were not considered. Blood samples were collected in acid/citrate/dextrose by venipuncture. All subjects gave informed consent.

Assay of acetylcholinesterase

Each blood sample was centrifuged for 15 min at 1000 *g*, the plasma and buffy coat were removed, and the resulting erythrocytes were washed 4–5 times with 0.154 M-NaCl. Acetylcholinesterase activity was assayed in red cells following the method of Beutler [11] based on the procedure of Ellman *et al.* [12]. Haemoglobin was estimated by the ferricyanide/cyanide method as described by Beutler [11]. Acetylcholinesterase activity is expressed in i.u. (1 i.u. = μmol of acetylthiocholine iodide hydrolysed/min) per g of haemoglobin at 37 °C. A molar absorption coefficient of 1.36×10^{-4} litre·mol^{-1}·cm^{-1} was used for the thionitrobenzoate ion at 412 nm.

Blood glucose values were determined by using an Ames Glucometer from Miles Laboratories Inc., Elkhart, IN, U.S.A.

Statistical analysis of the data was performed by employing Student's *t* test, a probability of 0.001 being used as the level of significance.

RESULTS

The erythrocyte membrane acetylcholinesterase activities in normal, diabetic and insulin-treated patients are shown in Table 1. The activity of the enzyme was observed to be significantly ($P < 0.001$) decreased in the diabetic condition; however, normal activity of the enzyme was restored after treatment with insulin *in vivo*. For the sake of brevity, the erythrocyte acetylcholinesterase activity of only a group of diabetic subjects (fasting blood glucose 13.8 ± 0.33 mM, mean ± S.D.) is shown in Table 1. However, in the present study, patients with different degrees of diabetic control through insulin injections were taken (as indicated by their fasting blood glucose levels) and their erythrocyte membrane acetylcholinesterase activity was plotted against the fasting blood glucose concentration (Fig. 1). The results show a

Table 1. Erythrocyte membrane acetylcholinesterase activity in normal, diabetic and insulin-treated patients

Enzyme activity is expressed in terms of i.u. (μmol of acetylthiocholine iodide hydrolysed/min) per g of haemoglobin at 37 °C. All values are means ± S.D.

Condition	n	Fasting blood glucose level (mM)	Acetylcholinesterase activity (i.u/g of haemoglobin)
Normal	8	4.4±0.27	36.66±0.59
Type 1 diabetic	11	13.8±0.33	12.24±0.82
Insulin-treated	5	4.8±0.17	37.54±1.14

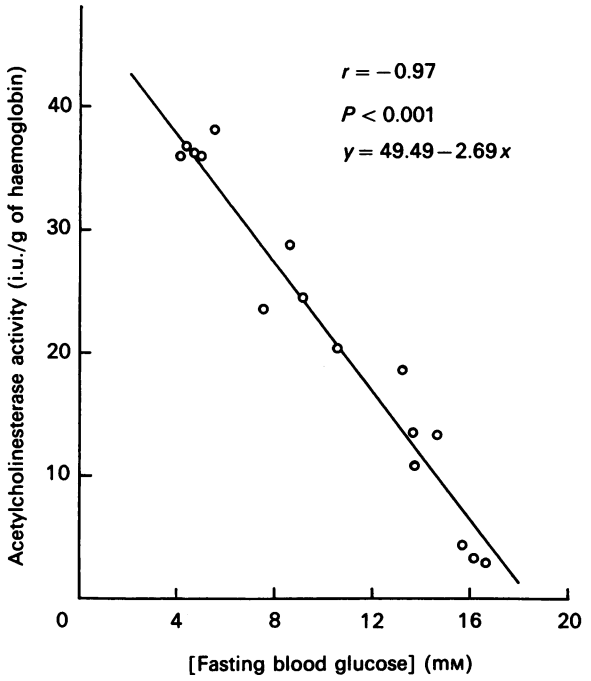

Fig. 1. Correlation between erythrocyte membrane acetylcholinesterase activity and fasting blood glucose level in type 1 diabetic patients

The acetylcholinesterase activity was determined in diabetic patients under varying degrees of control through insulin injection as indicated by their fasting blood glucose level. The enzyme activity is expressed in i.u./g of haemoglobin at 37 °C. All points are the means of at least three independent experiments.

significant negative correlation ($r = -0.97$) between the enzyme activity and the fasting blood glucose concentration. To characterize further the decreased acetylcholinesterase activity observed in type 1 diabetic patients, some kinetic parameters of the enzyme were determined. Acetylcholinesterase activity of normal (fasting blood glucose 4.23±0.13 mM; $n = 3$), diabetic (16.07±0.55 mM; $n = 3$) and insulin-treated diabetic (4.97±0.25 mM; $n = 3$) subjects was measured as a function of acetylthiocholine iodide concentration. The data were then plotted as the double reciprocal transformation [12a] of the Michaelis–Menten relation for the determination of K_m and $V_{max.}$ (Fig. 2). The mean value of $V_{max.}$ for the

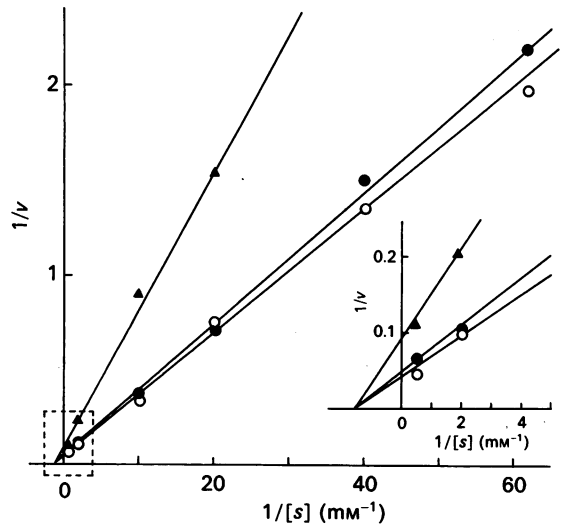

Fig. 2. Lineweaver–Burk plot of erythrocyte membrane acetylcholinesterase in normal, type 1 diabetic and insulin-treated diabetic conditions

Acetylcholinesterase activity of erythrocyte membranes at 37 °C is plotted as a function of acetylthiocholine iodide concentration. The intercept on the ordinate is equal to $1/V_{max.}$ and the intercept on the abscissa is equal to $-1/K_m$ (○, normal; ▲, diabetic; ●, insulin-treated diabetic). The method of least squares has been used to fit our data to the Lineweaver–Burk plot. The inset shows the expanded portion of boxed area of the plot.

enzyme with acetylthiocholine iodide as substrate was considerably decreased in diabetes (normal, 25.0 μM; diabetes mellitus, 10.87 μM; insulin-treated diabetics, 22.22 μM); however, the K_m values were unchanged.

DISCUSSION

Our results clearly show that the activity of the enzyme acetylcholinesterase in the erythrocyte membrane is significantly decreased in type 1 diabetes mellitus, and the decrease was observed to have a negative correlation with the fasting blood glucose level ($r = -0.97$), which reflects the severity of the disease. The results agree with the findings of Agarwal *et al.* [13] showing a reduction of acetylcholinesterase activity in alloxan-induced diabetic rats. Insulin treatment *in vitro* has been shown to stimulate acetylcholinesterase activity in slices of rat brain [2] and isolated erythrocyte membrane [13], which agrees with our findings of the restoration of normal activity of the enzyme in insulin-treated subjects.

The interaction of insulin with the erythrocyte-membrane receptor is now a subject of general interest, and binding of insulin has been shown to cause a change in the membrane fluidity [14]. Juhan-Vague *et al.* [15] reported that erythrocyte membranes obtained from uncontrolled type 1 diabetic patients have an increased membrane fluidity compared to normal, and fluidity returns to normal after insulin treatment. Insulin has already been reported to decrease membrane fluidity in the rat erythrocyte membrane [16]. Massa *et al.* [17] have shown that the value of the Hill coefficient (h) for acetylcholinesterase in erythrocyte membranes changes inversely in response to insulin. According to our findings, the severity-dependent decrease in the activity of this enzyme in erythrocytes of diabetics could thus be

explained, in part, by changes in fluidity characteristics of the membrane in response to low insulin levels in the diabetic condition.

It has been reported that acetylcholinesterase activity is influenced by membrane surface phenomena [18]. A decrease in the erythrocyte membrane sialic acid content has been reported in diabetes [8], and since sialic acid constitutes the principal charged component of the membrane, its loss from the erythrocyte surface in the diabetic condition 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 [19,20]. Obviously this would affect lipid–protein interactions. Given the decreased $V_{max.}$ of erythrocyte-membrane acetylcholinesterase in diabetes, it can be assumed that such a mechanism would influence the kinetic properties of the enzyme.

Regardless of the exact mechanism(s) by which insulin produces its effects on acetylcholinesterase activity, these findings clearly show that the acetylcholinesterase activity correlates with the blood glucose level, and this may be due to a concentration-dependent effect of insulin. Similarly, the number of active enzyme molecules on the erythrocyte surface may be controlled by insulin, since its deficiency in the diabetic condition is manifested by a decreased $V_{max.}$ of the enzyme. It is worth mentioning here that reticulocytes lack acetylcholinesterase activity, and the enzyme activity appears during erythrocyte maturation [21]. The restoration of normal activity after insulin treatment poses an impediment in explaining the mode of insulin action in modulating acetylcholinesterase. Whether insulin alters the membrane microenvironment of this enzyme by changing membrane fluidity, or whether it helps in maintaining the number of active enzyme molecules, is not fully understood. Furthermore, some of the properties of acetylcholinesterase present in brain tissue and erythrocytes are reported to be similar [3], and insulin is suggested [22–25] to play a potential role as the neurotransmitter or neuromodulator in brain tissue. In light of the above reports, our findings may help to explain the neuronal complications taking place in diabetes mellitus.

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