Biochemical changes in erythrocyte membrane in uncontrolled type 2 diabetes mellitus

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Received 4 October 2004; revised 5 July 2005

The levels of lipid peroxidation and alterations in lipid composition and ATPase activities were determined in erythrocyte plasma membrane of uncontrolled type 2 diabetes mellitus (DM) patients. The study groups consisted of 30 patients (16 males, 14 females) attending the Out Patients’ Department of Lokmanya Tilak Municipal General Hospital, Mumbai, and 23 age- and sex-matched control subjects (15 males, 8 females). Glycated haemoglobin (an index of long-term glycaemic control), erythrocyte membrane cholesterol, phospholipid and cholesterol to phospholipid molar ratio, lipid peroxidation products in the form of thiobarbituric acid-reactive substances (TBARS), and Na⁺-K⁺-ATPase activity were found to be significantly increased, and Mg²⁺-ATPase activity significantly decreased, in the diabetic subjects, as compared to controls. The study suggests that biochemical changes in the erythrocyte membrane may be involved in the pathophysiology of type 2 DM.

Keywords: Uncontrolled type 2 diabetes mellitus, erythrocyte membrane, glycated haemoglobin, Na⁺-K⁺-ATPase, Mg²⁺-ATPase, lipid peroxidation, cholesterol, phospholipid

IPC Code: A61K38/42; A61P7/00

Several abnormalities, such as reduced life span¹, increased viscosity², excessive aggregation³, and increased tendency to adhere to endothelial cells⁴ have been reported in the erythrocytes of diabetes mellitus (DM) patients. Changes in levels of phospholipids⁵, cholesterol⁶, cholesterol to phospholipid molar ratio⁷, unsaturated fatty acids⁸ and altered membrane phospholipid asymmetry⁹ have also been reported in the erythrocyte membranes of diabetic patients. Alterations in lipid composition can affect the physico-chemical properties of the erythrocyte membrane⁹, including plasma membrane Na⁺-K⁺-ATPase (E.C. 3.6.3.9) and Mg²⁺-ATPase (E.C. 3.6.3.1) activities¹⁰. A decrease in the plasma membrane Na⁺-K⁺-ATPase activity has been observed in erythrocyte membranes of diabetics¹¹. Insulin, within a physiological range of concentration normalizes lipid composition and order in erythrocytes in diabetic patients¹². It stimulates Na⁺-K⁺-ATPase activity and translocation to plasma membrane via phosphorylation of the α subunits by protein kinases¹³.

Glucose under physiological conditions produces oxidants that possess reactivity similar to hydroxyl free radicals¹⁴. Increased free radical production and elevated levels of lipids and several peroxidation products have been reported in diabetic patients¹⁵,¹⁶. Erythrocytes are exposed to continuous oxidative stress, because oxygen radicals are continuously generated by the auto-oxidation of haemoglobin¹⁷. Oxygen radicals formed over and above the detoxifying capacity of erythrocytes can cause peroxidative breakdown of phospholipid fatty acids and accumulation of malonyldialdehyde¹⁸. Erythrocytes of diabetic patients are more susceptible to lipid peroxidation, when treated with hydrogen peroxide in vitro¹⁹.

The present study was undertaken to determine levels of lipid peroxidation and alterations in lipid composition and ATPase activities in erythrocyte plasma membrane of uncontrolled type 2 DM patients.

Materials and Methods

Subjects and sample collection

The study group consisted of uncontrolled type 2 DM patients (n=30; 16 males, 14 females) attending the Out Patients’ Department of Lokmanya Tilak Municipal General Hospital, Mumbai. They were freshly diagnosed diabetics and were not under medication for the disease previously. Age- and sex-matched control subjects (n=23; 15 males, 8 females)
were clinically non-diabetic and normal. After an overnight fast, the blood was drawn from a forearm vein in 0.1 M trisodium citrate (0.5 mL/4.0 mL blood), sodium fluoride (4.0 mg 1:3 (w/w) sodium fluoride:potassium oxalate/mL blood) and plain glass ampules. All samples were immediately processed.

**Isolation of erythrocyte membranes**

Erythrocyte membranes were isolated as described\(^2^0\). The procedure was carried out in cold conditions. Briefly, the blood collected in 0.1 M trisodium citrate was centrifuged at 3000 rpm for 15 min, and the plasma and buffy coat was aspirated off. Saline-washed erythrocytes were lysed in 10 vols of ice-cold 5.0 mM Tris-HCl (pH 7.0), 1.0 mM EDTA (pH 7.0). The resulting erythrocyte membranes were resuspended twice in ice-cold lysis solution, centrifuged at 6000 rpm for 15 min, suspended in ice-cold 0.05 M Tris-HCl (pH 7.0), 1.0 mM EDTA, 0.5 M NaCl (pH 7.0) and centrifuged at 6000 rpm for 15 min. Treatment of membranes with the high NaCl concentration renders them free of most of the residual haemoglobin. The faint pink membranes, thus obtained were suspended in ice-cold 5.0 mM Tris-HCl (pH 7.0), 1.0 mM EDTA (pH 7.0) and centrifuged at 6000 rpm for 15 min. The resulting haemoglobin-free milky-white erythrocyte membranes were suspended in 0.05 M Tris-HCl (pH 7.0). Protein in erythrocyte membranes was estimated by the method of Lowry et al.\(^2^1\).

**Assay of ATPase activity**

ATPase activity in erythrocyte membranes was assayed by a modification of the previously described method\(^2^2\). Briefly, the membrane suspension was diluted to a volume containing ~150-200 µg protein/100 µL membrane suspension for the ATPase assay. ATP neutralised to pH 7.0 by 100 mM Tris was used for the assay. ATPase activity was expressed as µmoles P\(_i\)/mg erythrocyte membrane protein/60 min.

For total ATPase activity, erythrocyte membranes (~150-200 µg protein/100 µL membrane suspension) were incubated at 37°C for 60 min in 0.5 mL reaction mixture containing 2.0 mM ATP, 100 mM NaCl, 20 mM KCl, 5.0 mM MgCl\(_2\), 1.0 mM EDTA in 50 mM Tris-HCl (pH 7.0), and for Mg\(^2+\)-ATPase activity, in 0.5 mL reaction mixture containing 2.0 mM ATP, 5.0 mM MgCl\(_2\) in 50 mM Tris-HCl (pH 7.0). The reaction was stopped by adding 0.1 mL of 10% sodium dodecyl sulphate (SDS). Inorganic phosphorus (P\(_i\)) hydrolyzed from the reaction was measured as described\(^2^3\). ATPase activity was calculated by the difference between 0 min (reaction stopped immediately with SDS) and 60 min incubation periods. Na\(^+\)-K\(^+\)-ATPase activity was calculated from the difference between total ATPase and Mg\(^2+\)-ATPase activities.

**Erythrocyte membrane parameters**

Thiobarbituric acid-reactive substances (TBARS) in erythrocyte membranes were estimated as described\(^2^4\). Lipids were extracted from erythrocyte membrane\(^2^5\) using chloroform:methanol (2:1) mixture and cholesterol\(^2^6\) and phospholipids\(^2^7\) were determined as described.

**Determination of glycated haemoglobin (GHb), plasma and serum parameters**

GHb was determined by an adaptation of the thiobarbituric acid (TBA) method\(^2^8\). Plasma glucose was measured by the glucose oxidase-peroxidase method (Eco-Pak, Accurex Biomedical Pvt. Ltd., Mumbai). Total lipids\(^2^9,3^0\) and cholesterol\(^2^6\) in serum were measured as described. Na\(^+\) and K\(^+\) in serum were determined using a flame photometer (Mediflame 127, Systronics, Ahmedabad).

**Statistical analyses**

Significance of the differences was determined by Student’s \(t\) test\(^3^1\). A ‘\(P\)’ value greater than 0.01 was considered non-significant.

**Results and Discussion**

The clinical and erythrocyte membrane characteristics of study subjects are given in Tables 1 and 2, respectively. The diabetic male subjects showed significantly higher concentrations of erythrocyte membrane cholesterol (\(P<0.001\)), phospholipids (\(P<0.001\)) and TBARS (\(P<0.025\)), as compared to age- and sex-matched controls. Similarly, diabetic female subjects had significantly higher concentrations (\(P<0.01\)) of cholesterol and TBARS, but phospholipids concentration was not significantly different (\(P<0.5\)). The cholesterol to phospholipids molar ratio in erythrocyte membranes of both male and female diabetics was significantly higher (\(P<0.001\)), as compared to controls. No statistically significant difference was found in serum cholesterol and total lipids levels between diabetic and age- and sex-matched control subjects. However, increased erythrocyte membrane lipid concentrations in
diabetics suggest that changes in erythrocyte membrane lipid composition may accompany poor glycaemic control in patients, presenting with apparent normo-lipidaemia.

TBARS have been shown to be elevated in patients with diabetes. The generation of reactive oxygen species (oxidative stress) may play an important role in the aetiology of diabetic complications. The univalent reduction of oxygen results in a series of cytotoxic oxygen species, such as superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH). Studies of human erythrocytes in vitro and rat and rabbit erythrocytes in vivo have shown that membrane lipid peroxidation results in decreased cell survival, altered membrane phospholipid asymmetry, hypercoagulability, and increased adhesion to endothelial cells.

Both the diabetic male and female subjects had significantly higher ($P<0.001$) erythrocyte membrane lipid peroxidation activity and significantly lower ($P<0.001$) activity of $Na^+$$-$$K^+$$-ATPase$, as compared to age- and sex-matched controls. These results are in agreement with previous reports. Na$^+$$-$$K^+$$-ATPase$ activity, which accounts for ~40% of total ATPase activity in erythrocytes from normal subjects, accounts for ~30% in diabetic subjects. In contrast, Mg$^{2+}$$-ATPase$, which accounts for ~60% of total activity in controls, accounts for ~70% in diabetic patients, in agreement with previous report.

Type 2 diabetic subjects included in the present study were primarily from the lower socio-economic strata and may have had undetected DM for a long period of time, before presenting with hyperglycaemia and being diagnosed as diabetics. Thus, even newly

### Table 1—Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Male subjects</th>
<th>Female subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>48.93±9.38</td>
<td>50.06±8.11</td>
</tr>
<tr>
<td>Plasma glucose (g%)</td>
<td>92.8±12.05</td>
<td>167.6±57.52*</td>
</tr>
<tr>
<td>Glycated haemoglobin (%)</td>
<td>3.81±1.02</td>
<td>7.89±2.13*</td>
</tr>
<tr>
<td>Serum cholesterol (g%)</td>
<td>160.17±35.7</td>
<td>193.67±32.13#</td>
</tr>
<tr>
<td>Serum total lipids (g%)</td>
<td>282.16±57.88</td>
<td>338.33±94.49f</td>
</tr>
<tr>
<td>Na$^+$ (mEq/L)</td>
<td>132.46±3.33</td>
<td>143.37±3.73*</td>
</tr>
<tr>
<td>K$^+$ (mEq/L)</td>
<td>4.56±0.32</td>
<td>5.68±0.67*</td>
</tr>
</tbody>
</table>

*$P<0.001$; #$P<0.025$; f$P<0.1$; ‡$P<0.2$; +$P<0.5

### Table 2—Erythrocyte membrane characteristics of study subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Male subjects</th>
<th>Female subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (µg/mg protein)</td>
<td>125.19±23.93</td>
<td>170.36±20.13*</td>
</tr>
<tr>
<td>Phospholipids (µg/mg protein)</td>
<td>11.93±2.09</td>
<td>14.45±1.13*</td>
</tr>
<tr>
<td>Cholesterol:phospholipids (mol/mol)</td>
<td>0.83±0.03</td>
<td>0.95±0.09*</td>
</tr>
<tr>
<td>TBARS (ng/mg protein)</td>
<td>80.74±13.79</td>
<td>96.93±18.83#</td>
</tr>
<tr>
<td>Total ATPase</td>
<td>1.05±0.02</td>
<td>1.1±0.02*</td>
</tr>
<tr>
<td>Na$^+$$-$$K^+$$-ATPase</td>
<td>0.39±0.02</td>
<td>0.29±0.03*</td>
</tr>
<tr>
<td>Mg$^{2+}$$-ATPase</td>
<td>0.66±0.03</td>
<td>0.81±0.02*</td>
</tr>
</tbody>
</table>

* $P<0.001$; # $P<0.025$; f $P<0.01$; ‡ $P<0.5
diagnosed diabetics might be suffering from complications, arising due to prolonged hyperglycaemia, which may explain the significant differences observed between the patients and age- and sex-matched controls.

In conclusion, the present study found abnormal erythrocyte membrane characteristics in uncontrolled type 2 DM patients, suggesting that these changes may be involved in the pathophysiology of type 2 DM.

References