Critical role of plasma C-peptide on control of ATP/ADP ratio of RBC

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Abstract
Diabetes mellitus is a disorder that contributes whole metabolism of body. The results of this process are a rapid depletion of the intracellular ATP pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction. The ATP/ADP ratio of erythrocytes among four groups of normal individuals (young & old), athletes’ subjects and diabetes patients are compared and relationship between ATP/ADP ratio and C-peptide level of plasma are determined. ATP/ADP level and plasma C-peptide in four groups [normal (young & middle age), athletes, diabetes] are measured that show a significant difference between groups (P-value<0.001). A significant positive correlation is found between RBC ATP/ADP content (r=0.716; P<0.001). In this study, a positive relationship between RBC ATP/ADP ratio and C-peptide is found. Based on the obtained results, higher released C-peptide through plasma may control the ratio of ATP/ADP in erythrocytes of different individuals.

Keywords: Diabetes, ATP, ADP, C-peptide, Red Blood Cell.

INTRODUCTION

Diabetes mellitus is a disease involving a deficiency in insulin synthesis and/or a defect in glucose receptors. As a result, glucose levels in the blood and tissues, such as lens, kidney, vascular endothelial cells and erythrocytes are elevated. It has been postulated that prolonged states of hyperglycemia lead to the development of clinical complications, such as retinopathy, neuropathy and nephropathy [1-2]. One may expect that beside the serious disruptions in metabolism, diabetes also affects energetic conversions [3-10]. Decreased lipid oxidation in the basal state of obese and type2 diabetes (T2D) was reported [11]. Moreover, a decreased activity of the electron-transport-chain (ETC) specifically in subsarcolemmal mitochondria in T2D patients compared with obese and lean subjects has also been reported [12]. Numerous studies have shown an altered gene expression (mRNA) of several proteins involved in the oxidation phosphorylation (OXPHOS) [13-15]. Abnormalities in the catalytic subunit of ATP-synthase (a key enzyme in mitochondrial OXPHOS) in skeletal muscle of patients with T2D patients compared to lean subjects have been observed [16]. Other investigation showed poly (ADP ribose) polymerase (PARP) that is a profuse nuclear enzyme of eukaryotic cells that has been implicated in response to DNA injury.

Free-radical- and oxidant-induced cell injury can involve the activation of PARP. Activation of PARP by single-strand DNA (ssDNA) initiates an energy-consuming cycle by transferring ADP ribose units from NAD+ to nuclear proteins. The result of this process is a rapid depletion of the intracellular NAD+ and ATP pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction [17-23]. Note that metabolic changes that make impaired immune function observed in diabetic patients is due to dysfunction in ATP production [24]. Adenine nucleotides play a key role in the energy metabolism of cells. Levels
of ATP, ADP and AMP reflect the rate of energy transformations in mature red blood cells, fueled mainly by glycolysis and the pentose cycle [25]. The adenine nucleotide pool necessary for normal activity of erythrocytes remains under the control of an elaborate set of enzymes [26], which includes three magnesium-dependent regulatory enzymes: hexokinase (EC.2.7.1.1), phosphofructokinase (EC.2.7.1.11) and pyruvate kinase (EC.2.7.1.40). Minor shifts in the level of allosteric activators and inhibitors may grossly affect the rate of enzyme reactions, between them glycolysis, leading indirectly to changes in the level of ATP, ADP and AMP [27] and in the adenylate energetic charge of erythrocytes [28].

ATP can affect thromboregulation and stimulate the immune cells responsible for asthma attacks [29, 30]. ATP (or its metabolite ADP) subsequently binds to endothelial P2Y1 receptors, resulting in vasodilation [31, 32]. It has been proposed that acidemia due to ketoacidosis sometimes seen in severe or untreated diabetics might cause elevation of glucose 6-phosphate levels in the erythrocytes of diabetics, because phosphofructokinase activity is inhibited by the lower pH [33]. In human erythrocytes, the uptake of 3-o-methyl-D-glucose was decreased when the concentration of intracellular ATP was decreased by ca2+ ionophore A23187 [34]. The uptake of 2-deoxy-D-glucose in cultured rat pancreatic β-cells was inhibited by decreased intracellular ATP concentration caused by streptozotocin and also by oligomycin and carbonyl cyanid m-chlorophenylhydrazone (cccp), known as a potent uncoupler [35]. Direct activation of the glucose transporter by physiological concentration of ATP was demonstrated in human Erythrocytes [36-38]. Accordingly, the aim of this study was to study comparison of ATP/ADP level of RBC among three groups of normal, athletes’ subjects and diabetes patient and then clarify relationship between ATP/ADP and C-peptide level of plasma.

MATERIALS AND METHODS

Studies with human subjects:

The subjects were fully informed of any risks and discomforts associated with the experiments before giving their informed written consent to participate. The studies conformed to the code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the Ethics Committee of Shariati Hospital, Tehran University, Iran. In the first study 40 diabetes patients (Type2 diabetes) were collected which were treating with insulin and at the time of the study were diagnosed as uncontrolled (HbA1C% 8.79±0.19). Condition comparison studies were carried out, we also collected RBC from 50 healthy volunteer. All control subjects were healthy volunteer living in the community, and none was acutely ill. None exhibited evidence of cardiac or chronic kidney disease and all were euthyroid with normal liver function tests and normal value for plasma urea, creatinine and electrolytes. Furthermore, healthy subjects were drug free and with a negative family history of diabetes mellitus or hypertension. The following parameters were determined in all blood samples which collected at 8 hour after overnight fasting: erythrocyte ATP, ADP content and plasma C-peptide level.

ATP assay method in RBC:

ATP was measured by luciferin-luciferase technique [39, 40, 41]. In which the amount of light generated by the reaction of ATP with recombinant luciferase is dependent on the ATP concentration. Sensitivity was augmented by addition of the D-luciferin to the luciferase. A, 50 µl sample of RBC, lysed with TCA 10%(trichloroaceticacid) and neutralized with KOH 1M and diluted with Hepes buffer 100 mM pH 7.8 (1:64), injected into a cuvette containing 10 µl luciferin (sigma), 10 µl Mgso4, 10 µl luciferase(1 mg/ml). The peak light efflux from cuvette to which either known ATP standards or samples are added was determined using a luminometer (Sirius tube Luminometer, Berthold Detection System, Germany), a ATP standard curve was obtained on the day of each experiment.

RBC ADP assay procedure:

ADP was measured by the coupled assay of pyruvate kinase with luciferin-luciferase technique[42], in which at first we injected 5 µl pyruvate kinase (1 mg/ml) into a cuvettes containing 50 µl RBC(lysed,neutralized and diluted), 5 µl PEP (phospho enol pyruvate 20 mM, sigma), 5 µl KCl and patience for 7 min since the
ADPs existed in the sample converted to ATP, then added 10 µl luciferin(10 mM), 10 µl luciferase (1 mg/ml) and 10 µl Mgso4. the peak light efflux from cuvette to which either known ADP standards or samples are added was determined using a luminometer, a ADP standard curve was obtained on the day of each experiment.

**C-peptide assay procedure:**
This assay accomplished by automated electrochemiluminescence. C-peptide kit is buied from Cobas (Roche). Inject 500 µl of sample into cup and placed cup into instrument’ racks then defined program for instrument [43, 44].

**Statistical Method:**
Statistical significance among experimental periods and groups was determined with analysis of variance, Tamhane test and Scheffe test for multiple comparisons, Bivariate correlation for relation between variables and regression for prediction. A P-value 0.05 or less was considered statistically significant. Results are reported as the means ± SEM.

**RESULTS**

**Figure-1:** the comparison of ATP/ADP level among 4 groups (diabetes, normal (Y=young, M=middle age) and athletes). The amount of 53, 66, 63, 67 are out of average.

Human subject studied. RBCs are taken 40 male Type 2 diabetes who were diagnosed according by American Diabetes Association Guide lie 2009 [45]. Some patient had a family history of diabetes. The average age of the patients was 60.5±1.7 years. The data for average patient with diabetes and control are summarized in table1. In addition, RBC of 50 healthy human volunteers [20 athletes, 30 Normals (20 youngs, 10 middle age)] with equal sex for all was sampled. Average ages for athletic individuals without medication and history of diabetes disease, was 27.7±0.61 and for normal control was 26.7±0.49 and 50 ±0.60 subsequently. ATP/ADP level between groups

In this study the control individuals divided to three groups, normal and athlete individuals. ATP/ADP level in four groups (Normal(Y) & Normal (M), athletes, Diabetes) was measured and analyzed with ANOVA test, the result showed a significant difference between groups (P value<0.001), the result are summarized in table2 and Fig. 1.
Table 1: Age and sex characteristics of studied subjects: Erythrocyte ATP/ADP and plasma C-peptide content.

<table>
<thead>
<tr>
<th></th>
<th>Normal (Y)*</th>
<th>Normal (M)**</th>
<th>Athletes</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Age (year)</td>
<td>26.7±0.60</td>
<td>50±0.60</td>
<td>27.7±0.6</td>
<td>60±1.7</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>BMIa</td>
<td>25</td>
<td>28</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>ATP/ADPb</td>
<td>3.08±0.0</td>
<td>2.4±0.0</td>
<td>4.96±0.4</td>
<td>1.26±0.0</td>
</tr>
<tr>
<td>C-peptide(ng/ml)c</td>
<td>7.59±0.0</td>
<td>5.4±0.3</td>
<td>8.4±0.36</td>
<td>2.39±0.1</td>
</tr>
<tr>
<td>HbA1C%</td>
<td>4.86±0.0</td>
<td>5.31±0.0</td>
<td>4.09±0.0</td>
<td>8.79±0.1</td>
</tr>
<tr>
<td>FBSd</td>
<td>84.05±2.0</td>
<td>102.5±2.0</td>
<td>76±0.94</td>
<td>172±5.9</td>
</tr>
</tbody>
</table>

a=Body Mass Index [weight/(height)^2] =kg/m^2, b = P value<0.001, c=P value<0.001, d=fasting blood sugar
* Y= young, ** M= middle age.

C-peptide level between groups:

In this study we measured C-peptide by Electrochemiluminescence technique, and result was analyzed with ANOVA test. As the difference variance between groups was not significant, data were analyzed with fisher and Scheffe test. The results are summarized in table 3 and Fig. 2. Relationship between ATP/ADP and C-peptide was analyzed with Pearson correlation that shows a 0.716 unit increase in C-peptide is accompanied with 1 unit increase in ATP/ADP level (table 4).

Correlation between RBC ATP/ADP content and C-peptide:

A significant positive correlation was found between RBC ATP/ADP content (r=0.716; P<0.001). In this study, a positive relationship between ATP/ADP and C-peptide is found, accordingly; a 0.716 unit increase in C-peptide is accompanied by 1 unit increase of ATP/ADP ratio (Fig. 3). Therefore, based on experimental measurements reported here, using B Coefficient a formula for prediction of ATP/ADP ratio could be obtained: ATP/ADP= 0.429 + 0.420 [C-peptide] (Table 5).
Table-2. Tamhan test for ATP/ADP group comparison, as shown in this table the difference between groups are significant (P-value <0.001).

<table>
<thead>
<tr>
<th>Tamhan (ATP/ADP)</th>
<th>Factor(I)</th>
<th>Factor(J)</th>
<th>Sig.*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal(Y)¹</td>
<td>Normal(M)²</td>
<td>0.000</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Normal(M)²</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal(Y)</td>
<td>Normal(old)</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Normal(M)²</td>
<td>Athletes</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* The mean difference is significant at the 0.05 level. 1=young, 2=middle age.

Table-3. Scheffe test for comparison between groups shows that c-peptide differences between Normal and athletes individuals are not significant.

<table>
<thead>
<tr>
<th>Scheffe (C-peptide)</th>
<th>Factor(I)</th>
<th>Factor(J)</th>
<th>Sig.*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal(Y)¹</td>
<td>Normal(M)²</td>
<td>0.000</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Normal(M)²</td>
<td>Athletes</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Normal(Y)</td>
<td>Normal(old)</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Normal(M)²</td>
<td>Athletes</td>
<td>0.272</td>
</tr>
</tbody>
</table>

* The mean difference is significant at the 0.05 level. 1=young, 2=middle age.

Table-4. Pearson correlation between ATP/ADP and C-peptide, the correlation between C-peptide and ATP/ADP is 0.716.

<table>
<thead>
<tr>
<th></th>
<th>ATP/ADP &amp; C-peptide</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson correlation</td>
<td>0.705*</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.01 level (2-tailed).

DISCUSSION

Impairment in glucose metabolism in diabetic patients when it goes into cell is a main problem in treatment of diabetic patients [46]. It has been suggested that decrease in ATP/ADP ratio in pancreatic-β cells makes decrease in insulin and C-peptide secretion into plasma and led to type 2 diabetes [47]. The present study investigates the simultaneous effects of exercise and C-peptide level on the RBC metabolism rate, ATP/ADP content. In healthy control (normal and athletes) and type 2 diabetes (Fig.1, Table 2), a positive significant correlation (Fig.3, Table 4) between plasma C-peptide and ATP/ADP content of RBC among four groups has been observed. This observation

Figure-3. Correlation between RBC ATP/ADP content and C-peptide level between four groups.
suggests higher metabolic rate (ATP/ADP ratio) in athletes in comparison with normal and type2 diabetes. Other study reported that athletes have high ATP/ADP content in comparison with normal subject [48]. Also a similar positive correlation between exercise and erythrocyte Na+, K+-ATPase has been reported [49]. Indeed, Rabini et al. [50] and Petruzzi et al. [51] showed an increase in ATP concentration in erythrocytes in patients with the Type I diabetes while Na+, K+ ATPase activity that determines the intracellular ATP concentration was decreased. Moreover, other evidences reported that dysfunction of Na+-K+ ATPase and Ca2+-ATPase activity in the erythrocyte membrane is not directly connected with the degree of diabetic control because there is no correlation between enzymatic activity and fructose amine or glycemia in diabetic rats [52]. This enzymatic dysfunction is probably connected with the decreased ATP concentration, showed by the positive correlation between ATP concentration and the activity of Na+-K+ ATPase [49]. Many evidences suggested that Na+, K+ ATPase increases K+ concentration of cytoplasm and export Na+ into plasma which is accompanied by increase of cytoplasmic calcium [53]. Calcium itself activates calmodulin and calmodulin-dependent proteins such as CAMK, calcineurine [54, 55].

Through a cascade of signals these proteins activate metabolism and increase in ATP/ADP in cells [56, 57]; there are reciprocal relationship between ATP/ADP and Na+, K+-ATPase [58]. On the other hand, other evidences showed that dysfunction in Na+, K+-ATPase increases cytoplasmic concentration of Na+ and this ion led to calcium to be exited from mitochondria. It is known that some of enzymes in mitochondria such as dehydrogenases need calcium ion and this deficiency can led to impairment in mitochondria activity [59]. In this study, we report that released C-peptide from pancreatic cells activates metabolism in RBC and increases ATP/ADP ratio (Fig.3-4, table 4). In fact, measurements of plasma C-peptide by electrochemiluminescence and concomitant measurements of ATP/ADP level of the same individuals RBC by bioluminescence method [60-61] confirmed a direct relationship. C-peptide affects on a G-protein receptor and thereby resulted in an increase in Na+, K+-ATPase activity [62]. Different evidences implicate on increase of intracellular calcium by C-peptide [62, 63]. Interestingly, C-peptide stimulated specifically the classical PKC-α, calcium-dependent mediator of Na+, K+-ATPase phosphorylation in tubular cells [64]. These observations interpret strong reasons for increase in ATP/ADP content upon increase in plasma C-peptide. It is well known that the properties of diabetic erythrocytes are abnormal [65, 66]. These abnormalities include decreased deformability [67, 68], increased membrane viscosity [69] and increased erythrocyte aggregation [45, 70]. The decreased Na+, K+-ATPase activity observed in the diabetic erythrocyte membrane...
leads to an intracellular accumulation of sodium with subsequent accumulation of free calcium ions due to competition. A well-known reason for ATP/ADP low level in diabetic subject is due to anaerobic pathway of glucose metabolism in RBC which as mentioned earlier, dysfunction in mitochondria and pH decreasing can led to prevent the activity of some of glycolytic enzymes such as phosphofructokinase, hexokinase, pyruvate kinase [63,71].

In conclusion, according to the result obtained in this investigation; ATP/ADP ratio is high in athletes and normal individuals in comparison with diabetic patients. It seems that ATP/ADP content may be considered as a suitable parameter for diabetes control and also for athlete’s individuals as the higher ATP/ADP ratio can be suggested as a doping test similar to erythropoietin (EPO) effect because ATP/ADP ratio determines metabolic rate in individuals. However, it may be suggested that complementary C-peptide-insulin (digested proinsulin) for drug treatment to diabetes subject, as C-peptide increases ATP/ADP content in cells.

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