Purine nucleotides and their metabolites in erythrocytes of streptozotocin diabetic rats

W Dudzinska, AJ Hlynczak

SUMMARY

Objectives: In the present study it was tried to obtain a complete overview of purine nucleotide metabolism in erythrocytes of streptozotocin (STZ) induced diabetes mellitus rats.

Methods: Erythrocyte levels of the main nucleotides (ATP, ADP, AMP, GTP, GDP, GMP, IMP, NAD+, NADP+), nucleosides (Ado, Guo, Ino) and the base Hyp were measured using the HPLC method. The parameters that can be deduced from their concentrations: TAN, TGN and AEC, GEC expressed by the ratio of high/low energy nucleoside phosphates were calculated. The effects of streptozotocin-induced diabetes on the concentration and metabolism of rat erythrocyte purine and pyridine nucleotides and the activity of Na+K+-ATPase as well as Ca2+-ATPase were investigated.

Results: Increased dephosphorylation of adenine nucleotides (found as the increased concentration of Ado and Hyp and the decrease in AEC value) and the decrease in ATP and TAN and the changes in the concentrations of NAD+ and NADP+ suggest serious purine and pyridine metabolism disruptions in diabetic erythrocytes and decrease in ATPases activity.

Conclusion: The observations suggest that purine nucleotide degradation is markedly accelerated in erythrocytes of STZ diabetic rats.

Key-words: Streptozotocin-diabetic rats · Purine and pyridine nucleotides · ATPase · HPLC.

Dudzinska W, Hlynczak AJ. Purine nucleotides and their metabolites in erythrocytes of streptozotocin diabetic rats
Diabetes Metab 2004;30,557-567

RÉSUMÉ

Nucléotides des purines et leur métabolites dans les érythrocytes de rats rendus diabétiques par streptozotocine

Objectifs : Ce travail s’est fixé pour but de réaliser une étude complète du métabolisme des nucléotides des purines dans les érythrocytes des rat rendus diabétiques par streptozotocine (STZ).


Résultats : Une déphosphorylation accrue d’adénine nucléotides (délittue de l’augmentiation de concentration constatée de Ado et Hyp et de la baisse de valeur d’AEC) et la baisse d’ATP et de TAN et les changements des concentrations de NAD+ et NADP+ suggèrent de sérieuses anomalies du métabolisme des purines et pyridines des érythrocytes de rat rendus diabétiques et une baisse d’activité des ATPases.

Conclusion : Ces observations suggèrent une dégradation accélérée des purines nucléotides dans les érythrocytes de rats rendus diabétiques par STZ.

Mots-clés : Diabète induit par streptozotocine · Purine et pyridine nucléotides · ATPase · HPLC.

Address correspondence and reprint requests to:
W Dudzinska. Department of Biochemistry, Faculty of Natural Sciences, University of Szczecin, 3a Felczaka, 71-412 Szczecin, Poland.
wiola@univ.szczecin.pl
Received: April 26th, 2004; revised: October 20th, 2004
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 besides the serious disruptions in metabolism, diabetes also affects energetic conversions.

The study of purine nucleotide metabolism is very important for understanding of disruptions in energy metabolism as the purine nucleotides participate in most energy-requiring metabolic reactions and act as coenzymes. Purine nucleotide metabolism in erythrocytes is based on two fundamental pathways: the salvage pathway and the catabolic pathway. The salvage pathway can readily form mononucleotides from purine bases and their nucleosides. HGPRT catalyzes the formation of IMP from hypoxanthine (Hyp) and GMP from guanine (Gua). APRT catalyzes the conversion of adenine (Ade) to AMP. HGPRT returns the major products of purine nucleotide catabolism to nucleotide forms. Since the erythrocytes do not exhibit xanthine oxidase (EC 1.2.3.2) activity, Hyp is the end product of ATP degradation in these cells. Thus, adenine nucleotide catabolism can be measured as Hyp formation in erythrocytes [3–8]. In erythrocytes adenosine (Ado), guanosine (Guo) and inosine (Ino) can be phosphorylated to AMP, GMP, IMP, respectively [9–11]. Catabolic pathway of erythrocytes is involved in the following activities: AMP-D, 5’-NT, ADA, PNP [5, 7, 12–14].

Purine metabolism can be studied by the evaluation of the intracellular content. Although study of purine nucleotide metabolism in diabetes is not limited by the availability of cells, our knowledge in this field is still incomplete. Literature reports the activity of some specific enzymes in diabetes (AK, ADA, 5’-NT, AMP-D) [15–19]. There have been some studies on erythrocyte glucose, ATP, lactate concentrations and their modifications induced by plasma normal subjects [20–21]. A defect in the ATP re-release by osmotic shock was studied in erythrocytes from Type 1 diabetic patients [22]. Some reports concern the relations between the intracellular concentration of ATP and the activity of membrane ATPases [23–27]. However, the reports are not fully consistent.

ATP plays a significant role in transferring energy within the cell, diffusing from where it is produced to where it is utilized. ATP in erythrocytes is utilized in ion transport processes that account for almost 30% of total ATP consumption. Na⁺ and K⁺ transport utilizes much more ATP, while Ca²⁺ transport requires markedly less energy. There is no consensus in the literature on the dependence of Na⁺,K⁺-ATPase and Ca²⁺-ATPase on ATP. That is why we also attempted to find out the relations between the ATP concentration and the activity of Na⁺,K⁺-ATPase (EC 3.6.1.37) and Ca²⁺-ATPase (EC 3.6.1.3).

In the present study we tried to obtain a complete overview of purine nucleotide metabolism in erythrocytes of streptozotocin induced diabetes mellitus rats. First we measured erythrocyte levels of the main nucleotides (ATP, ADP, AMP, GTP, GDP, GMP, IMP, NAD⁺, NADP⁺), nucleosides (Ado, Guo, Ino) and the base Hyp with the HPLC. We calculated the parameters that can be deduced from their concentrations: TAN, TGN and AEC, GEC respectively [15–19]. To investigate the effects of streptozotocin-induced diabetes on concentration and metabolism of rat erythrocyte purine and pyridine nucleotides and the activity of Na⁺,K⁺-ATPase and Ca²⁺-ATPase.

Materials and methods

Animals. Experimental diabetes

The studies were carried out on 25 male Wistar rats, body weight 280–380 g. The animals came from the Immunology and Experimental Therapy PAN Institute in Wroclaw, Poland. The rats were maintained in accordance with standard laboratory animal procedures. During the experiment they were fed a standard rat chow diet and had free access to water ad libitum.

Diabetes was induced by tail vein single injection of 55 mg/kg body weight streptozotocin (STZ). STZ was dissolved in 1 mL 10 mM citrate buffer, pH 4.5. Control rats were injected with citrate buffer instead of STZ. Blood sam-
samples for glucose measurements were taken from the tail vein about 48 h after STZ injection.

All the animals were weighed before and on the 10th day after the injection. On the 10th day of the experiment the animals were anesthetized with intraperitoneal pentobarbital sodium, 40 mg/kg b.w., then killed by decapitation and the blood was removed. Rats with blood glucose concentration of 13.9 mM or more were considered diabetic. The experimental groups included control (n = 10) and diabetic rats (n = 15).

Chemicals

Purines used as chromatographic standards were obtained from Sigma-Aldrich. HPLC-grade potassium dihydrogen orthophosphate, potassium chloride and tripotassium orthophosphate were obtained from Fluka Chemie GmbH. HPLC-grade acetonitrile was obtained from Merck. HPLC-grade water was purified on two steps (reverse osmosis and ion exchanger) Mili-Q-System of Millipore. All solvents used for HPLC determinations were filtered through 0.22 μm nylon filters (Supelco). Streptozotocin was obtained from Sigma-Aldrich. Blood glucose levels were measured by a glucose oxidase method using a commercial reagent test kit (bioMérieux). Serum fructosamine was determined using a commercial reagent test kit (bioMérieux). Serum glucose levels were measured by a glucose oxidase method using HPLC chromatography data system run on Chemist Software for Windows 98.

Instrumentation

Glucose analyses were performed on Technicon RA-500 analyzer. Serum fructosamine analyses were performed on Cobas DP 25 Photometer analyzer.

A Hewlett-Packard Series 1100 apparatus was used for HPLC analysis. It consisted of a quaternary pump system with a degasser and continuous seal wash option (G1311A) and thermostatted column compartment (G1316A). The analytical column measuring 100 mm × 4.6 mm was packed with 18.3 μm Hypersil BDS-C (Hewlett Packard). The samples were introduced using a Rheodyne 7725 injection valve equipped with a 20 μL loop. Sample peaks were integrated and quantified using HPLC chromatography data system run on Chemist Software for Windows 98.

HPLC separation of purine

The samples (500 μL) of heparinized blood were deproteinized with an equal volume of 1.3 M perchloric acid in 1.5 mL Eppendorf tubes and centrifuged at 16000 g for 10 min, at 4 °C. The supernatant (600 μL) was neutralized with 60-90 μL of 3 M potassium orthophosphate solution to a pH within 6.0-7.0. The neutralized extract was again centrifuged and filtered through a 0.22 μm nylon filter. The clear filtrate was then used for HPLC assay or stored at -80 °C until analysis.

Chromatographic conditions

The purine and pyridine nucleotides; ATP, ADP, AMP, GTP, GDP, GMP, IMP, NAD+, NADP+; nucleosides Ado, Guo, Ino and the bases Hyp were separated with the HPLC method of Smolenski et al. [28]. Sample aliquots (100 μL) were injected into the chromatography column. The nucleotides were separated using a linear phosphate buffer gradient system (buffer A: 150 mM KH₂PO₄, 150 mM KCl adjusted to pH 6.0 with K₂HPO₄; buffer B: 15% v/v solution of acetonitrile in buffer A) at a flow rate at 0.666 mL/min. The peaks were detected by absorption measurements at 254 nm. The composition of the mobile phase was controlled by low-pressure gradient mixing device. The cycle time was 12.8 min between injections. The analytical column was maintained at constant temperature 20.5 °C.

Preparation of hemoglobin free erythrocyte ghost membranes

Hemoglobin free erythrocyte ghost membranes were prepared by the procedure of Cha et al. [29]. Heparinized erythrocytes were washed four times with six times their volume of 0.9% NaCl, and after centrifugation the white buffy layers overlaying the erythrocytes was eliminated as completely as possible. After the last washing 1 volume of packed erythrocytes was hemolized in 5 volumes of solution containing 1 mM EDTA and 1 mM Tris-HCl (pH 7.4) buffer. About 15 min after hemolysis, the whole homogenate was centrifuged at 20000 g for 15 min. The precipitate was sequentially washed twice with 3 volumes of the lysing solution, once with 3 volumes of 10 mM Na₂EDTA solution and once again with 3 volumes of the lysing solution. This precipitate was washed once with 3 volumes of 2.0% NaCl solution, 4 times with 0.5 mM histidine-imidazole buffer, pH 7.0, and was finally suspended in 0.5 mL, at room temperature. The serum and buffy layers were removed by aspiration to obtain packed erythrocytes which were washed at 4 °C with histidine-imidazole buffer, pH 7.0.

Protein concentration of erythrocyte membrane preparations was determined according to Lowry et al. [30] using bovine serum albumin as a standard.

Measurement of ATPase activities

The Na⁺,K⁺-ATPase and Ca²⁺-ATPase activities were determined on erythrocyte plasma membranes by the method of Choi et al. [31]. For determination of membrane ATPase activities, aliquots of 25 μL membrane suspension, containing 25-50 μg of protein, were preincubated at 37°C.
for 5 min in the following media: (1) Na⁺,K⁺-ATPase: 20 mM KCl, 100 mM NaCl, 0.5 mM EGTA, 30 mM histidine-imidazole buffer, pH 7.0, 5 mM MgCl₂. (2) Ca²⁺-ATPase: 120 mM KCl, 0.3 mM CaCl₂, 30 mM histidine-imidazole buffer, pH 7.0, 5 mM MgCl₂. The enzymatic reaction was started by adding 2 mM ATP. After 30 min of incubation at 37°C reaction was stopped by adding 1 mL of ice-cold 20% trichloric acid. Then the mixture was centrifuged at 20000 g for 2 min and measurement of the Pi (by standard procedure using ammonium molybdate and ascorbic acid) released into the supernatant was carried out according to the method of Goldberg and Fernanden [32].

Enzyme activity is expressed as of nmol of P₁ per mg membrane proteins per 60 min.

Statistical analysis

The Statistica v.5.1 software was used for the statistical analysis. A non-parametric test for small or uneven size groups was used to assess variables regardless of its distribution. The significance of differences between groups was tested with the non-parametric ANOVA Kruskal-Wallis tests. Since the data were not generally normally distributed according to the Shapiro-Wilk’s test (except the clinical and biochemical parameters) a test for Spearman rang correlation coefficient (rₛ) was used to check the statistical significance of observed correlations.

Results

The studies on experimental diabetes were carried out according to the model of the STZ-induced diabetes. This model reflects the Type 1 diabetes [33] and is one of the most common experimental models for studying the insulin-dependent diabetes [34-35]. The studies were carried out 10 days after the STZ injection, with acute disease symptoms [36]. Therefore, the observed changes in the studied group exclude the contribution of the insulin increase to purine metabolism regulation and ATPases activity.

The mean glucose concentration in the blood from the animals with the streptozotocin-induced diabetes on the 10th day of the experiment was significantly higher (p < 0.01) compared with a control group. Fructosamine concentration in serum also significantly increased (p < 0.01) in diabetic rats. A 12% decrease in body weight of the diabetic rats was evident after 10 days of diabetes, with a 4% increase in the control group (Tab I). High concentrations of glucose and fructosamine, as well as the decrease in body weight, indicate diabetes in the studied rats.

The ATP, ADP, AMP stoichiometrically couple all of the metabolic sequences of a living cell. The amount of metabolically available energy that is momentarily stored in the adenylate system is linearly related to the mole fraction of ATP plus half the mole fraction of ADP; this parameter has been termed the AEC of the adenylate pool. AEC is thus ((ATP+0.5 [ADP])/([ATP]+[ADP]+[AMP]).

The absolute intracellular ATP concentration depends on the TAN and the AEC, that is, on the fraction of ATP in the TAN. TAN was calculated from the median concentrations [ATP], [ADP] and [AMP]. The concentration of ATP in erythrocytes in diabetic rats was significantly lower than in the control rats by 20% (Tab II). ATP concentration correlated negatively with glucose concentration in whole blood and fructosamine in serum (rₛ = -0.52), Figure 1. ADP concentration did not change significantly in the diabetic rats compared with the control group. Table II also shows the differences in the content of AMP for experimental group. Diabetic rats showed a significant decrease (about 23%) of erythrocyte AMP with respect to control group. TAN in erythrocytes diabetic rats was significantly lower than in the control group by 17% (Tab III). Also AEC values decreased (Tab III). No correlation was found between TAN and glucose or fructosamine concentrations, as well as between AEC and these parameters. The decrease in ATP and AMP concentrations, as well as in TAN and AEC values, shows significant alterations in the concentration of adenine nucleotides in diabetes.

The concentrations of GTP and GDP in erythrocytes in diabetic rats were significantly lower than in the control rats by 12% and 32%, respectively (Tab II). No significant

### Table I

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 10)</th>
<th>Diabetic rats (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mM, 10 days after STZ injection)</td>
<td>x̄ 6.4</td>
<td>26.3*</td>
</tr>
<tr>
<td>SD</td>
<td>1.06</td>
<td>2.84</td>
</tr>
<tr>
<td>Serum fructosamine (mM, 10 days after STZ injection)</td>
<td>x̄ 1.2</td>
<td>2.9*</td>
</tr>
<tr>
<td>SD</td>
<td>0.05</td>
<td>0.31</td>
</tr>
<tr>
<td>Body weight (g, 1 days after STZ injection)</td>
<td>x̄ 324.0</td>
<td>325.0</td>
</tr>
<tr>
<td>SD</td>
<td>41.15</td>
<td>33.91</td>
</tr>
<tr>
<td>Body weight (g, 10 days after STZ injection)</td>
<td>x̄ 339.5</td>
<td>287.5*</td>
</tr>
<tr>
<td>SD</td>
<td>40.51</td>
<td>32.63</td>
</tr>
</tbody>
</table>

The values are expressed as mean SD. *P < 0.01 with respect to values in control group.

n = number of cases.

The blood glucose (mM) and serum fructosamine (mM) were measured on the 10th day after STZ injection.

Glucose analyses were performed on Technicon RA-500 analyzer. Serum fructosamine analyses were performed on Cobas DP 25 Photometer analyzer.
changes were found in GMP concentration. TGN calculated from the median concentrations \([\text{GTP}] + [\text{GDP}] + [\text{GMP}]\) in erythrocytes of diabetic rats was significantly lower than in the control group by 13\% (Tab III). No significantly differences were found in GEC \((\frac{[\text{GTP}]}{[\text{GTP}] + [\text{GDP}] + [\text{GMP}]})\) (Tab III).

The concentration of IMP in erythrocytes was significantly higher in the STZ-induced diabetic rats. IMP level was 34\% higher compared with the control group (Tab II).

Ino in diabetic rats was not significantly different from that of the controls (Tab IV).

A significant increase in Ado and Guo concentration in blood was seen in diabetic rats (Tab IV). No correlation was found between AMP and Ado concentrations and GMP as well as between these parameters and glucose or fructosamine concentrations.

Adenine nucleotide catabolism can be measured as Hyp formation. Table IV also shows the differences in the con-

### Table II

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 10)</th>
<th>Diabetic rats (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATP</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>1193.88</td>
<td>983.57**</td>
</tr>
<tr>
<td>min. - max.</td>
<td>1129.33 - 1386.50</td>
<td>700.62 - 1270.34</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>1169.53 - 1292.11</td>
<td>748.33 - 1193.71</td>
</tr>
<tr>
<td><strong>ADP</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>220.17</td>
<td>235.60</td>
</tr>
<tr>
<td>min. - max.</td>
<td>168.74 - 288.02</td>
<td>201.35 - 313.31</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>169.93 - 263.30</td>
<td>222.47 - 251.92</td>
</tr>
<tr>
<td><strong>AMP</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>42.36</td>
<td>32.18*</td>
</tr>
<tr>
<td>min. - max.</td>
<td>15.44 - 72.13</td>
<td>19.22 - 42.53</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>35.46 - 59.73</td>
<td>28.01 - 34.40</td>
</tr>
<tr>
<td><strong>GTP</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>133.66</td>
<td>118.84*</td>
</tr>
<tr>
<td>min. - max.</td>
<td>116.45 - 163.09</td>
<td>105.22 - 165.27</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>126.94 - 151.63</td>
<td>113.96 - 125.78</td>
</tr>
<tr>
<td><strong>GDP</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>74.11</td>
<td>51.25*</td>
</tr>
<tr>
<td>min. - max.</td>
<td>39.79 - 91.12</td>
<td>40.53 - 63.95</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>51.24 - 82.60</td>
<td>44.89 - 55.41</td>
</tr>
<tr>
<td><strong>GMP</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>9.65</td>
<td>8.80</td>
</tr>
<tr>
<td>min. - max.</td>
<td>2.54 - 21.24</td>
<td>5.40 - 15.52</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>7.74 - 10.94</td>
<td>7.11 - 12.37</td>
</tr>
<tr>
<td><strong>IMP</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>12.76</td>
<td>20.37*</td>
</tr>
<tr>
<td>min. - max.</td>
<td>6.23 - 27.04</td>
<td>14.03 - 29.48</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>10.13 - 19.20</td>
<td>15.99 - 21.86</td>
</tr>
<tr>
<td><strong>NAD+</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>251.73</td>
<td>225.80*</td>
</tr>
<tr>
<td>min. - max.</td>
<td>226.36 - 273.29</td>
<td>174.85 - 279.60</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>242.66 - 263.06</td>
<td>217.69 - 241.04</td>
</tr>
<tr>
<td><strong>NADP+</strong> ((\text{mmol/L RBC}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>27.33</td>
<td>35.06**</td>
</tr>
<tr>
<td>(Q_{25} - Q_{75})</td>
<td>23.66 - 28.67</td>
<td>32.34 - 36.88</td>
</tr>
</tbody>
</table>
The ATP was separated with the HPLC method of Smolenski and measured on the 10th day after STZ injection. Serum fructosamine analyses were performed on Cobas DP 25 Pho-photometer analyzer. Glucose analyses were performed on Technicon RA-500 analyzer.

Hyp as the products of the adenine nucleotide degradation (indicated by the increased concentration of Ado and Hyp with respect to control) and the decrease in AEC) and the decrease in TAN suggest serious energy metabolism disruptions in diabetic erythrocytes. The increased dephosphorylation of adenine nucleotides (indicated by the increased concentration of Ado and Hyp as the products of the adenine nucleotide degradation and the decrease in AEC) and the decrease in TAN suggest serious energy metabolism disruptions in diabetic erythrocytes.

The coenzymes NAD+/NADH and NADP+/NADPH are redox pair of pyridine nucleotides: their biological effects are determined by the redox state. NAD⁺ and its derivatives NADH, NADP⁺ and NADPH have regulatory functions in the generation of triose phosphates and pyruvate from glucose. NAD⁺/NADH catalyzes reactions of glycolysis and sorbitol pathway in the cytosol. The redox pair NADP⁺/NADPH regulates steps of the sorbitol and pentose phosphate pathway.

The concentration of NAD⁺ in erythrocytes in diabetic rats was significantly lower while the concentration of NADP⁺ was significantly higher. The decrease in NAD⁺ was 10% while the increase in NADP⁺ was 33% (Tab II).

The activity of Na⁺,K⁺-ATPase in diabetic was lower than in the control rats. Similarly, Ca²⁺-ATPase activity was also lower (Tab II). The positive correlation was found between the ATP concentration and Na⁺,K⁺-ATPase activity (rs = 0.53) (Fig 2).

Discussion

Our studies on the purine metabolism of erythrocytes in diabetic rats showed significant alterations in the concentration of purine nucleotides and their derivatives.

Changes in adenine and guanine nucleotide concentration are difficult to explain. They depend on many factors that do not exist separately but are complementary. Apart from the impact of changes in purine enzyme activity, the changes in adenine and guanine nucleotide concentration may result from mechanisms responsible for the glucose metabolism. Increased glucose conversion on the glycolytic pathway, as well as the increased glucose utilization [20-21, 23, 37-39] could suggest the increased ATP concentration in erythrocytes in diabetes. Indeed, Rabini et al. [26] and Petruzzi et al. [22] showed an increase in ATP concentration in erythrocytes in patients with the Type 1 diabetes as a result of decreased Na⁺,K⁺-ATPase that according to the authors determines the intracellular ATP concentration. Possible explanations for this finding might be an increased ATP synthesis caused by the administered insulin [40-41], almost always in supraphysiological amount, during Type 1 diabetes treatment, and a reduced Na⁺,K⁺-ATPase activity which is the major ATP-hydrolyzing enzyme of the erythrocyte. The changes in the rats examined in the present study exclude the contribution of the insulin increase to purine metabolism regulation and ATPase activities.

In the present study the activity Na⁺,K⁺-ATPase and Ca²⁺-ATPase was lower in diabetic rats. This decrease in Na⁺,K⁺-ATPase activity and Ca²⁺-ATPase activity in the erythrocyte membranes is not directly connected with the degree of diabetic control because there is no correlation between enzymatic activity and fructosamine or glycemia in the diabetic rats. This enzymatic dysfunction is probably connected with the decreased ATP concentration, indicated by the positive correlation between ATP concentration and the activity of Na⁺,K⁺-ATPase. Similar correlation was observed by Rabini et al. [25]. The present study shows that diabetic rats had lower ATP concentration in erythrocytes compared with the control group. The results agree with data reported for streptozotocin diabetic rats [24, 42] and type 2 diabetes subjects [22]. One explanation for the lower ATP content could be the requirement for protein kinase or the ATP-dependent K⁺/Cl⁻ [43] but this has not been investigated in the present study. On the other hand our observation that ATP was inversely correlated with the levels of glycemia might indicate decreased ATP production under
Purine in streptozotocin-diabetic rats

Hyperglycemic conditions. It is interesting, in this regard, that Eto et al. [44] observed decreased ATP and increased glucose-6-phosphate content in diabetic hamsters of the CHAD strain myocardial.

Table III
Total adenine (guanine) nucleotides TAN (TGN) and adenylate (guanylate) charge AEC (GEC) values of erythrocytes in control and streptozotocin-diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 10)</th>
<th>Diabetic rats (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAN (mmol/L RBC)</td>
<td>median 1486.00</td>
<td>1250.19*</td>
</tr>
<tr>
<td></td>
<td>min. - max. 1369.30 - 1732.26</td>
<td>956.36 - 1619.49</td>
</tr>
<tr>
<td>AEC</td>
<td>median 0.90</td>
<td>0.88*</td>
</tr>
<tr>
<td></td>
<td>min. - max. 0.86 - 0.94</td>
<td>0.83 - 0.91</td>
</tr>
<tr>
<td>TGN (mmol/L RBC)</td>
<td>median 212.51</td>
<td>181.60*</td>
</tr>
<tr>
<td></td>
<td>min. - max. 185.92 - 252.64</td>
<td>151.39 - 243.55</td>
</tr>
<tr>
<td>GEC</td>
<td>median 0.80</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>min. - max. 0.74 - 0.86</td>
<td>0.75 - 0.84</td>
</tr>
</tbody>
</table>

TAN (TGN) = [ATP] ([GTP]) + [ADP] ([GDP]) + [AMP] ([GMP]). AEC was evaluated according to the formula by Atkinson: ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]); GEC = ([GTP] + 0.5[GDP])/([GTP] + [GDP] + [GMP]). The values of TAN and TGN are expressed in mmol/L RBC as median, min.-max., Q25-Q75. *P < 0.05 with respect to values in control group.

Table IV
Purine and pyridine nucleosides content in control and streptozotocin-diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 10)</th>
<th>Diabetic rats (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ado</td>
<td>median 0.72</td>
<td>2.75***</td>
</tr>
<tr>
<td>(mmol/L whole blood)</td>
<td>min. - max. 0.24 - 1.37</td>
<td>1.52 - 5.35</td>
</tr>
<tr>
<td>Guo</td>
<td>median 0.46</td>
<td>5.69**</td>
</tr>
<tr>
<td>(mmol/L whole blood)</td>
<td>min. - max. 0.40 - 0.47</td>
<td>3.97 - 8.36</td>
</tr>
<tr>
<td>Ino</td>
<td>median 4.28</td>
<td>3.39</td>
</tr>
<tr>
<td>(mmol/L whole blood)</td>
<td>min. - max. 1.59 - 11.32</td>
<td>2.01 - 5.20</td>
</tr>
<tr>
<td>Hyp</td>
<td>median 0.90</td>
<td>5.66*</td>
</tr>
<tr>
<td>(mmol/L whole blood)</td>
<td>min. - max. 0.27 - 6.76</td>
<td>3.80 - 11.49</td>
</tr>
</tbody>
</table>

The values are expressed in mmol/L whole blood as median, min.-max., Q25-Q75. **P < 0.0007 with respect to values in control group. ***P < 0.00001 with respect to values in control group. *P < 0.0015 with respect to values in control group.

Under physiological conditions, even small variations in ATP concentration lead to changes in AMP concentration, obeying the adenylate kinase equilibrium. However, in our studies the decrease in ATP concentration did not lead to...
the increase in ADP and AMP concentration; AMP concentration decreased. Such changes could only be explained by the disruptions on the pathway of adenine nucleotide resynthesis or degradation. It has been proven that the amount of PRPP, a direct substrate in the reaction catalyzed by APRT and HGPRT, is lower in erythrocytes with the pyruvate kinase deficiency (PK; EC 2.7.1.40). Sitzmann [45] demonstrated a decrease in the activity of PK in Type 1 diabetes. The inhibition of the PK activity in erythrocytes leads to the decrease in ATP, PRPP and NAD+ concentration [46]. The decrease in ATP concentration is not accompanied by an increase in ADP and AMP concentrations in the red cells [47-48]. Adenosine pathway is also inhibited [48].

The observed higher adenosine concentration in blood provokes a question about its role in the resynthesis of adenine nucleotides on the adenosine pathway in diabetic erythrocytes. In the present study we showed that diabetic rats had higher Ado concentration in blood. Ado has been reported to be a potent antagonist of insulin action in various tissues. [49]. In addition, the sensitivity of several tissues to Ado, including kidney, heart and brain increases as a result of insulin-dependent diabetes [50]. Therefore, elevated (even locally) Ado concentrations would have important physiological consequences, especially at the early stage of diabetes.

Changes in Ado concentration depend on the activity of the enzymes metabolizing this nucleoside and on the transport through the plasma membranes with a help of Na+-dependent and Na+-independent transporters. In diabetes Ado transport to cells is lower because of the lower amount of the nucleoside transporter [19]. The increase of Ado concentration in tissues and blood in diabetes results from lower level of transcript for the genes of transporters ENT1 and ENT2 [51].

Since the main way of removing adenosine from blood is its uptake by the erythrocytes [10, 52-54], the significant increase in adenosine concentration observed in our studies puts forth a question about the role of that nucleoside in adenylate metabolism in diabetes. It has been reported that the increased adenosine concentration increases the concentration of adenine nucleotides in erythrocytes [5]. Komarova et al. [10] observed an increase in ATP and TAN after adding adenosine into the erythrocyte hemolysate. According to these authors, ATP and TAN are dependent on the adenosine concentration and the kinetic characteristics of the AK which activity increases in proportion with the supply of adenosine. Ateullakhanov et al. [9] after stimulating erythrocytes in vitro to utilize ATP (through the increase in ATP-dependent Na+,K+-ATPase activity) showed that the exogenic adenosine at the concentration of 0.4-0.8 mM also allowed for the reinstatement of the adenine nucleotide pool. Reports by Kim [53] and Komarova et al. [10] suggest the participation of adenosine in the resynthesis of the adenine nucleotide pool, especially at P nucleotides. The stimulating effect of P nucleotides on the ATP synthesis was also confirmed by Plagemann et al. [52]. The results of the studies indicate the participation of P nucleotides in the stimulation of the AK activity.

The increased Ado concentration in diabetic rats in the present study, with a simultaneous decrease in ATP and AMP (in consequence decrease in TAN and AEC) may
suggest impairment of nucleotide resynthesis on the adenosine pathway. Adenosine pathway requires AK activity that is inhibited in diabetes [19, 50]. However, the increase in Ado concentration and decrease in AMP seems not only result from the decrease in AK activity. For example, Valentine et al. [55] showed that an increase in ADA results in the decreased TAN. ATP is depleted (and in consequence TAN) as a result of ADA inhibition [56].

Erythrocytes need highly active AMP-D to stabilize their energy charge. If the energy charge decreases for some reason, the AMP concentration rises. AMP-D degrades AMP, thereby increasing the energy charge. Oxidative stress present in diabetes even in normoglycemia, and a subsequent higher free radicals concentration as well as lower activity of the anti-oxidative enzymes in erythrocytes in diabetes may modify the activity of enzymes connected with adenine nucleotide metabolism, e.g. AMP-D [57-58]. Perhaps the decrease in ATP and AMP concentrations in diabetic rats comes from the lowered activity of the enzyme catalyzing phosphorylation of Ado into AMP [19, 50], but it also may result from the higher activity of the enzymes catalyzing deamination of AMP and Ado [16-17, 54, 57-58], which, together with the observed decrease in AEC, may indicate the intensification of purine nucleotides catabolism. A similar trend was observed in the distribution of GMP concentrations (decrease of concentration) and guanosine (increase), which could suggest a lowered activity of guanosine kinase and higher activity of enzymes connected with GMP catabolism.

Our study shows that in diabetes ATP and AMP decrease, as well as the values of TAN and AEC. It could suggest disruption on the adenine nucleotide resynthesis pathway. Moreover, we observed a decrease in NAD+ and increase in NADP+. A correct course of glycolysis depends largely on the NAD+ concentration and resynthesis because NAD+ is necessary to oxidize gliceraldehyde-3-phosphate which in turn enables the resynthesis of ATP [59]. Some reports and our results indicate the changes in the concentrations of the studied pyridine nucleotides. However, there is no full consistency in the reports. Velikij et al. [60] showed 50% decrease in NAD+ and NADP+ in liver and the lens of diabetic rats. Glock and McLean [61] showed the decrease of these nucleotides in liver but not in the diaphragm. Song et al. [62] measured the concentrations of pyridine nucleotides in erythrocytes in diabetic patients (Type 2) and reported the decreased NAD+ concentration and increased NADP+ concentration. The discrepancies may come from the fact that the concentration of the pyridine nucleotides was measured in different tissues and by means of various methods. The observed increase in NADP+ concentration may suggest that the activation of glucose conversions on the reductive pathway is connected also with the increase in NADP+ concentration and decrease in NADPH concentration [62-63]. Unless it is compensated by the increase in the activity on the pentose phosphate pathway, the lower ratio NADPH/NADP+ leads to the inhibition of the sorbitol synthesis [64]. It is therefore possible to suggest that a strong relation between the pyridine nucleotide metabolism and the pentose phosphate pathway conversions is very important in diabetes.

To sum up, the increased dephosphorylation of adenosine nucleotides (visible due to the increased concentration of Ado and Hyp as the products of their degradation and the decrease in AEC) and the decrease in ATP and TAN and the changes in the concentrations of NAD+ i NADP+ suggest serious purine and pyridine metabolism disruptions in diabetic erythrocytes and decrease in ATPases activity.

It is not clear whether the presented disruptions in purine metabolism in diabetic rats could be confirmed in Type 1 and 2 diabetes. The influence of insulin and the degree of metabolic control of diabetes on the studied parameters is unknown. These issues require further studies.

References

55. Valentine WN, Paglia DE, Tartaglia AP, Glisans F. Hereditary hemo-
lytic anemia with increased red cell adenosine deaminase and
56. Siaw MF, Mitchell BS, Koller CA, Coleman M, Hutton JJ. ATP
depletion as a consequence of adenosine deaminase inhibition in man.
57. Tavazzi B, Di Poro D, Amorini AM, et al. Energy metabolism and
lipid peroxidation of human erythrocytes as function of increased ox-
impairment of human erythrocyte energy metabolism through the
oxygen radical-mediated direct activation of AMP-deaminase. J Biol
59. Wahlberg G, Adamson U, Svensson J. Pyridine nucleotides in glucose
metabolism and diabetes: a review. Diabetes Met Res Rev, 2000, 16,
33-42.
60. Velikiij NN, Obrosova IG, Efimov AS, Rabicheva EI, Sokil OP. Nicot-
inamide coenzymes in the regulation of cellular metabolism in vari-
61. Glock G, Mc Lean P. Effects of hormones on levels of oxidized and
reduced diphosphopyridine nucleotide and triphosphopyridine
62. Song H, Han X, Yuon H, Kang T, Wu H. Activities of aldolase reduct-
ase, ATPase, and nucleotide concentrations of erythrocytes in patients
with type 2 (non-insulin-dependent) diabetes mellitus. Chinese Med J,
and glutathione redox status in non-insulin-dependent diabetic
64. Grunewald RW, Weber I, Kinne RK. Control of sorbitol metabolism
in renal inner medulla of diabetic rats. Biochim Biophys Acta, 1993,
1225, 39-47.