Platelet Functions and Energy Metabolism in a Patient With Hexokinase Deficiency

By Jan-Willem N. Akkerman, Gert Rijksen, Gertie Gorter, and Gerard E. J. Staal

We have studied the regeneration of adenosine triphosphate (ATP) in the glycolytic pathway in platelets with a 75% reduction in hexokinase (HK) activity and have investigated aggregation and Ca$^{2+}$ secretion. HK-deficient platelets had a normal glycolytic flux in the resting state, but responded insufficiently to stimulation with thrombin (5 U/ml). In contrast, oxygen contents and glycanolysis were normal. When the metabolic adenine nucleotides were labeled with $^{14}$C-adenine, the patient's platelets showed a normal adenosine energy charge and a normal level of $^{14}$C-ATP. However, the inhibitor of mitochondrial energy generation, CN$^{-}$, induced a weaker fall in $^{14}$C-ATP in the patient's platelets than in the controls. Analysis of secretion markers revealed decreased amounts of granule-bound ATP and secreteable Ca$^{2+}$, whereas granule-bound adenosine diphosphate (ADP), β-thrombogloblulin, N-acytyl-β-o-glucosaminidase, and β-glucuronidase were within the normal range. Aggregation and Ca$^{2+}$ secretion induced by 5 U/ml thrombin were normal and were not changed in the presence of inhibitors of mitochondrial and glycogenolytic energy generation. Aggregation was also normal at 0.1 U/ml thrombin and was independent of these inhibitors, but Ca$^{2+}$ secretion was greatly impaired when mitochondrial and glycogenolytic ATP resynthesis was abolished. These findings indicate that a severe reduction in HK activity causes insufficient acceleration of the glycolytic flux during stimulation with thrombin. This leads to impaired dense granule secretion in conditions where secretion depends on concurrent ATP resynthesis and glycolysis is rate limiting.

**MATERIALS AND METHODS**

**Patient**

The proband, a 20-yr-old woman with nonspherocytic hemolytic anemia and severe hexokinase type I deficiency in red blood cells, lymphocytes, and platelets, has been described previously. The patient had no history of a bleeding tendency. The bleeding time (Simplate II, General Diagnostics, Morris Plains, NJ) was 2 min and 50 sec—well within the normal range (3–8 min, n = 20). Platelet count was increased (786,000 ± 187,000 cells/μl, mean ± SD, n = 6). Informed consent was obtained from the patient and control subjects.

**Platelet Isolation**

Freshly drawn venous blood (50-ml portions) was collected from the patient and from healthy volunteers into citrate (0.1 vol of 130 mM trisodium citrate). The donors had not taken any medicines for 10 days prior to blood collection. After centrifugation (200 g, 10 min, 22°C), the supernatant, platelet-rich plasma, was collected, and for some experiments, incubated with 1 μM [U-$^{14}$C]-adenine (specific activity 286 Ci/mole, Radiochemical Center, Amersham, U.K.) for 30 min at 37°C to label the metabolic pool of the adenine nucleotides. The platelets were isolated by passage through a Sepharose 2B column (Pharmacia, Uppsala, Sweden; column-size 2.5 cm x 15 cm) equilibrated in Ca$^{2+}$-free Tyrode’s solution (pH 7.2), with or without 5 mM glucose (see Results). The final concentration of platelets was standardized at 2.0–2.5 x 10$^8$ cells/ml by dilution in gel-filtration buffer. The gel-filtered platelets were prewarmed for 3 min at 37°C before the start of the metabolic and functional studies.

From the Departments of Haematology and Medical Enzymology, University Hospital Utrecht, Utrecht, The Netherlands. Submitted February 7, 1983; accepted July 22, 1983. Address reprint requests to Dr. J. W. N. Akkerman, Department of Haematology, University Hospital Utrecht, Catharijnesingel 101, 3511 GV Utrecht, The Netherlands. © 1984 by Grune & Stratton, Inc. 0006-4971/84/6301-0018$01.00/0

**Hexokinase (HK)**

HK catalyzes the phosphorylation of glucose to glucose-6-phosphate in the first reaction of the glycolytic pathway. In numerous tissues, HK is an important regulatory enzyme, especially since it works at the beginning of a long metabolic sequence. The regulatory role of HK is depicted by its rather low maximal activity compared to the glycolytic flux, its mass:action ratio that shows that the HK reaction in situ is displaced from equilibrium, its strong regulation by glucose-1,6-diphosphate and other metabolites, and by the serious consequences of a partial deficiency in red blood cells, where it causes severe nonspherocytic hemolytic anemia.

The role of HK in platelets is uncertain. Although its maximal activity is low indeed, it is difficult to calculate the ratio of products over substrates of the HK reaction in situ, since glucose is present below detection limits, and adenosine tri- and diphosphates (ATP and ADP) are distributed among different compartments. Rough calculations contradict an equilibrium reaction, but an additional problem is the compartmentation of HK with more than equilibrium reaction, but an additional problem is the activity bound to membranes, together with one of its severe deficiency of HK type I in red blood cells, lymphocytes, and platelets. In this report we describe the influence of this defect on platelet energy metabolism, aggregation, and dense granule secretion.
General Biochemical Parameters

Glycolytic enzymes, glycogen, and the contents of the secretory granules were analyzed immediately after gel filtration in glucose-containing medium. Hexokinase was measured at 37°C in an assay coupled to the glucose-6-phosphate dehydrogenase reaction after containing medium. Hexokinase was measured at 37°C in an assay grainules were analyzed immediately after gel filtration in glucose-oxidase-peroxidase system, using ABTS (2.2'-azino-di-3-ethyl-benzthiazoline sulfonate) (Boehringer, Mannheim, F.R.G.) as a redox indicator, and was expressed as mmole glycosyl residues/10^11 platelets. The contents of the secretion granules was investigated by measuring granule-bound ATP and ADP, endogenous serotonin, and secretable Ca^2+ ions as markers for dense granule contents; β-thromboglobulin as a marker for the α-granules; and N-acetyl-β-d-glucosaminidase and β-glucuronidase as markers for the acid hydrolases containing vesicles types I and II, respectively. ATP and ADP were measured in ethanol extracts using the luciferin-luciferase technique and a Packard Pico-lite luminometer (Packard Instrument Co. Downers Grove, IL). The metabolic pool of ATP-ADP was separated from the granule pool by controlled digitonin-induced lysis of gel-filtered platelets, followed by discontinuous gradient centrifugation, as described previously. An extra treatment in this study was the addition of 8 mM EDTA to the cell suspension immediately before the digitonin treatment in order to prevent ATP hydrolysis after cell disruption. The data were corrected for nonlysed cells, which were recovered in the pellet fraction (based on lactate dehydrogenase). The recovery of this procedure was 95%; the variation coefficients for 8 determinations in normal subjects. In the patient, however, the combination of CN and antimycin-A had the same effect on lactate generation and analysis of 14C-ATP and its derivatives.

RESULTS

General Biochemical Parameters

Platelet HK activity in the patient was about 25% of the normal average, as reported previously. The other glycolytic enzymes tested (phosphoglucone isomerase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase), as well as glucose-6-phosphate dehydrogenase, were within the normal range (data not shown). Glycogen content was normal. Total ATP and ADP were similar in platelet-rich plasma (data not shown) and gel-filtered platelets (Table 1). Both fell within the normal range, but ATP was relatively low, which prompted us to investigate the contents of this nucleotide in the metabolic pool and the granule compartment. Subcellular fractionation by digitonin revealed that the metabolic pool was normal. Granule-bound ATP, however, was strongly decreased, suggesting the presence of a slight storage pool deficiency in the dense granules. In agreement with this observation was the low amount of secretable Ca^2+ ions. Granule-bound ADP and endogenous serotonin, however, were not decreased. β-Thromboglobulin was low, but still within the normal range. The activities of N-acetyl-β-d-glucosaminidase and β-glucuronidase were normal (Table 1).

Carbohydrate Metabolism

The HK-deficient platelets had a normal production of lactate in the absence of metabolic inhibitors (Table 2). Inhibition of mitochondrial ATP resynthesis by 1 mM CN (Table 2) or 8.25 μg/ml antimycin-A (not shown) induced a 1.5-fold increase in lactate production, which again was normal. This increase could be completely abolished by glucono-δ-lactone, indicating that the extra lactate generated in the presence of CN was the result of glycogen catabolism. Thrombin (5 U/ml) more than doubled the rate of lactate formation in normal platelets, but the increase in the HK-deficient platelets was only about 1.5-fold. The addition of CN led to a further activation in thrombin-treated normal platelets, but not in the HK-deficient platelets. Glucono-δ-lactone sharply reduced the rate of lactate production, both in the patient and in normal subjects. In the patient, however, the combination of CN and glucono-δ-lactone reduced the rate of lactate production almost to values found in resting platelets, whereas in normal platelets, the difference from the resting state was still about 1.5-fold.

Since platelets generate lactate both from glucose
catabolism was therefore studied in detail by measur-
mo. Data on AlP and ADP refer to ethanol-soluble nucleotides.

and from glycogen, the abnormalities found in lactate formation could be explained by a defect in glycolysis or glycogenolysis (or both). The patient's glycoly

Energy Metabolism

Unstimulated HK-deficient platelets maintained a normal balance between production and consumption of metabolic energy, as illustrated by the high AEC (patient 0.93 ± 0.01, n = 3; normals 0.92 ± 0.03, n = 3) and the constant level of 14C-ATP during 30-min incubation (Table 3). CN−, and especially the combination of CN− and glucono-δ-lactone, induced a transient imbalance between ATP regeneration and ATP consumption, leading to a fall in 14C-ATP and a concurrent accumulation of 14C-hypoxanthine-inosine (not shown) until a new equilibrium was established. The fall in 14C-ATP induced by CN− was significantly smaller in HK-deficient platelets than in normal platelets. When the platelets were stimulated with thrombin (5 U/ml) under the same conditions, the glycogen content remained constant during the first minute after stimulation and then decreased to 75% after 7.5 min and to 10% during the subsequent 60 min. A similar decrease was found in normal controls, indicating that, during stimulation with thrombin, glycogen was consumed at a normal velocity. It is therefore likely that HK-deficient platelets have a normal glycogen catabolism. The abnormalities found in the formation of lactate (Table 2) are thus indicative of a defect in the glycolytic pathway.

Table 2. Lactate Production in HK-Deficient Platelets

<table>
<thead>
<tr>
<th>Additions</th>
<th>Thrombin (5 U/ml)</th>
<th>Patient</th>
<th>Percent</th>
<th>Controls</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>2.13 ± 0.20 (3)</td>
<td>(100)</td>
<td>2.22 ± 0.17 (7)</td>
<td>(100)</td>
</tr>
<tr>
<td>CN−</td>
<td>-</td>
<td>3.05 ± 0.35 (3)</td>
<td>143</td>
<td>3.11 ± 0.26 (5)</td>
<td>140</td>
</tr>
<tr>
<td>CN− + gluconolactone</td>
<td>-</td>
<td>1.80 ± 2.30 (2)</td>
<td>96</td>
<td>2.10 ± 0.21 (4)</td>
<td>95</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>3.05 ± 3.55 (2)</td>
<td>155</td>
<td>5.00 ± 0.58 (5)</td>
<td>225</td>
</tr>
<tr>
<td>CN−</td>
<td>+</td>
<td>3.28 ± 0.41 (3)</td>
<td>154</td>
<td>5.41 ± 0.80 (5)</td>
<td>244</td>
</tr>
<tr>
<td>CN− + gluconolactone</td>
<td>+</td>
<td>2.17 ± 2.28 (2)</td>
<td>104</td>
<td>3.50 ± 0.34 (5)</td>
<td>158</td>
</tr>
</tbody>
</table>

Data are expressed as μmole lactate formed/min/10^11 cells and are ranges (n = 2) or means ± SD (n ≥ 3). Intervals between the determinations were 1–3 mo. CN− and glucono-δ-lactone were present at final concentrations of 1 mM and 10 mM, respectively.
Table 3. Energy Metabolism in HK-Deficient Platelets

<table>
<thead>
<tr>
<th>Incubation (min)</th>
<th>CN (1 mM)</th>
<th>14C-ATP Patient</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>83 ± 1 (3)</td>
<td>84 ± 4 (5)</td>
</tr>
<tr>
<td>30</td>
<td>–</td>
<td>82 ± 1 (3)</td>
<td>80 ± 5 (4)</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>69 ± 3 (3)</td>
<td>53 ± 6 (4)</td>
</tr>
</tbody>
</table>

Data on 14C-ATP are expressed as percentage of total 14C radioactivity (means ± SD, with number of determinations). Intervals between the determinations were 1–3 mo.

was steeper in the presence of CN− and when both CN− and glucono-δ-lactone were present. In general, these patterns were the same in HK-deficient and normal platelets. Similar findings were obtained with 5 U/ml thrombin, although here the fall in 14C-ATP and AEC was considerably steeper. Again, no differences were found between patient and controls (data not shown).

Aggregation and Ca2+ Secretion

HK-deficient platelets aggregated normally upon stimulation with a high (5 U/ml) or a low (0.1 U/ml) dose of thrombin. The aggregation patterns were the same in the presence of antimycin-A with or without glucono-δ-lactone (Fig. 1). The amount of Ca2+ secreted upon addition of 5 U/ml thrombin was subnormal, probably as a result of a slight storage pool deficiency (Table 1), and not changed by the presence of metabolic inhibitors at such a high thrombin concentration (Table 4). This was in sharp contrast with experiments performed with 0.1 U/ml thrombin. Without inhibitors, Ca2+ secretion was about 90% of maximal both in the patient and in the controls. Antimycin-A reduced the secretion by 24% (from 91% to 67%) in the patient platelets and only 10% (from 89% to 79%) in the controls. The effect was even greater when both inhibitors were present, where a reduction of 62% (from 91% to 29%) was found for the HK-deficient platelets compared to only 18% (from 89% to 71%) for the controls.

DISCUSSION

In this article we have evaluated the effect of a partial deficiency of platelet HK on the flux through the glycolytic pathway, the production of metabolic energy, and aggregation and Ca2+ secretion. Unstimulated HK-deficient platelets maintained a normal glycolytic flux, despite the 75% reduction in HK activity. When mitochondrial ATP resynthesis was blocked by CN−, the production of lactate increased 1.5-fold. This increase was inhibited by glucono-δ-lactone, and therefore, was mainly the result of the conversion of glycogen to lactate at a rate of approximately 0.5 μmole glycósyl residues consumed/min/10⁹ cells. In these respects, the HK-deficient platelets behaved...
HEXOKINASE-DEFICIENT PLATELETS

The HK-deficient platelets maintained a constant
\(^{14}\)C-ATP level and AEC during 30-min incubation, which accords with the normal glycogenolytic flux under the same conditions. The very low de novo synthesis, the lack of creatine-phosphate in human platelets, and the normal level of metabolic ATP and ADP (Table 1) make \(^{14}\)C-ATP and the AEC reliable indicators of the balance between production and consumption of metabolic energy.\(^{19}\) This balance was transiently disturbed by CN\(^{−}\) and glucono-\(\delta\)-lactone, which induced a fall in \(^{14}\)C-ATP and a concomitant increase in \(^{14}\)C-hypoxanthine-inosine. In many types of cells, including platelets, this ATP \(\rightarrow\) hypoxanthine conversion has been explained as an important mechanism that protects the cell from too excessive changes in the AEC.\(^{32,33}\) This mechanism is susceptible to inhibitors of mitochondrial and glycogenolytic ATP resynthesis, as depicted by the steeper fall in \(^{14}\)C-ATP in the presence of CN\(^{−}\) and glucono-\(\delta\)-lactone, but is apparently unaffected by a partial HK deficiency, since the fall in \(^{14}\)C-ATP is not steeper in the patient’s platelets than in the controls. Surprisingly, an opposite effect is seen when HK-deficient platelets are treated with CN\(^{−}\). Compared to a fall of 37% in controls, the fall in \(^{14}\)C-ATP in the patient was only 17%. The fall in \(^{14}\)C-ATP induced by CN\(^{−}\) is probably proportional to the extent to which the mitochondria contribute to ATP homeostasis. The observation that this fall is considerably smaller in the patient’s platelets than in controls would thus indicate that mitochondrial ATP resynthesis is impaired in HK-deficient platelets. This hypothesis would indicate that the total consumption of metabolic energy is reduced, since in resting HK-deficient platelets, ATP resynthesis in glycolysis and glycogenolysis appear normal (Table 2) and ATP production and consumption are balanced. HK is for 95% bound to membranes,\(^{23}\) probably the outer mitochondrial membrane,\(^{24}\) via a specific HK-binding protein and is thought to play a role as adenine nucleotide translocator.\(^{35,36}\) This implies a regulatory role for HK in the balance between aerobic and anaerobic energy generation, and the abnormalities found in \(^{14}\)C-ATP following CN\(^{−}\) addition could thus directly result from an impaired HK activity.

HK-deficient platelets showed a normal aggregation response upon stimulation, with a high (5 U/ml) or a low (0.1 U/ml) dose of thrombin. These patterns were not affected by antimycin-A or glucono-\(\delta\)-lactone. For Ca\(^{2+}\) secretion, different results were obtained, depending on the amount of thrombin used for stimulation. With 5 U/ml thrombin, which induces maximal secretion, HK-deficient platelets secreted slightly less Ca\(^{2+}\) than the controls. This difference was probably the result of a reduced amount of Ca\(^{2+}\) present in the dense granules where Ca\(^{2+}\) is stored together with granule-, nonmetabolic ATP and ADP. The low levels of granule ATP found in this study are in line with this observation and indicate a slight storage pool deficiency in the patient’s platelets. Recent observations suggest that storage pool deficiency is caused by impaired ATP translocation across the dense granule membrane.\(^{37}\) The fact that the same defect is found in HK-deficient platelets is of special interest, in view of the aforementioned role of HK in nucleotide translocation. At 5 U/ml thrombin, Ca\(^{2+}\) secretion was not affected by antimycin-A and glucono-\(\delta\)-lactone, which contrasts sharply with experiments performed with 0.1

Table 4. Ca\(^{2+}\) Secretion in HK-Deficient Platelets

<table>
<thead>
<tr>
<th>Additions</th>
<th>Thrombin (U/ml)</th>
<th>(\mu\text{M} \text{Ca}^{2+}/10^{11} \text{ Cells} )</th>
<th>Percent</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.0</td>
<td>7.9</td>
<td>(100)</td>
<td>13.4 ± 1.7 (14)</td>
</tr>
<tr>
<td>Antimycin-A</td>
<td>5.0</td>
<td>6.0</td>
<td>91</td>
<td>11.9 ± 1.6 (6)</td>
</tr>
<tr>
<td>Antimycin-A + gluconolactone</td>
<td>5.0</td>
<td>7.7</td>
<td>98</td>
<td>11.8 ± 1.8 (4)</td>
</tr>
<tr>
<td>None</td>
<td>0.1</td>
<td>5.3</td>
<td>67</td>
<td>10.6 ± 2.2 (6)</td>
</tr>
<tr>
<td>Antimycin-A</td>
<td>0.1</td>
<td>2.3</td>
<td>29</td>
<td>9.5 ± 1.9 (5)</td>
</tr>
<tr>
<td>Antimycin-A + gluconolactone</td>
<td>0.1</td>
<td>2.3</td>
<td>29</td>
<td>9.5 ± 1.9 (5)</td>
</tr>
</tbody>
</table>

Data of the patient are means of duplicate experiments and give a representative example of three studies. Data of the controls are means ± SD, with number of determinations.
U/ml thrombin. Here, Ca\textsuperscript{2+} secretion was normal in the absence of inhibitors, but greatly impaired by the combination of antimycin-A and glucono-δ-lactone. Interestingly, the initiation of the secretion response was not changed by the inhibitors. Under similar conditions, these inhibitors had much less effect on normal platelets. These observations suggest strongly that energy generation is directly linked to secretion of dense granule contents. The defect in HK activity becomes apparent only when ATP resynthesis via mitochondrial respiration and glycolgenolysis is inhibited. The fact that Ca\textsuperscript{2+} secretion is normal in the absence of metabolic blockade suggests that, under normal conditions, mitochondrial respiration and glycolgenolysis compensate for a reduction in glycolytic activity. Other studies\textsuperscript{38,39} have indicated that platelet aggregation and secretion are directly coupled to the availability of metabolic energy in which the velocities of these functions determine whether or not concurrent ATP resynthesis is essential. At a high dose of thrombin (5 U/ml), the extra energy needed for aggregation and Ca\textsuperscript{2+} secretion is obtained from rapid consumption of the cell's metabolic ATP and ADP contents, and concurrent ATP resynthesis is not required.\textsuperscript{38} At a low dose (0.1 U/ml), thrombin-induced platelet functions are much slower, and here, concurrent ATP resynthesis is essential for normal platelet functions to occur.\textsuperscript{38}

Our present results agree well with these observations and illustrate that secretion requires an intact ATP resynthesizing apparatus when induced by 0.1 U/ml thrombin, whereas rapid hydrolysis of existing metabolic ATP alone is sufficient for secretion at 5 U/ml thrombin. Under all conditions, HK-deficient platelets aggregate normally, which accords with the concept that aggregation requires less energy than secretion.\textsuperscript{39}

ACKNOWLEDGMENT

The authors gratefully acknowledge the cooperation of the patient. The assistance of Drs. D. Pott Hofstede and D. Westerink, Hospital "Het Diaconessenhuis," Hilversum, is highly appreciated.

REFERENCES

25. Akkerman JWN, Holmsen H, Loughnane M: Simultaneous measurement of aggregation, secretion, oxygen uptake, proton pro-
duction, and intracellular metabolites in the same platelet suspension. Anal Biochem 97:387, 1979


Platelet functions and energy metabolism in a patient with hexokinase deficiency

JW Akkerman, G Rijksen, G Gorter and GE Staal