Impaired Nicotinamide Adenine Dinucleotide Synthesis in Pyruvate Kinase–Deficient Human Erythrocytes: A Mechanism for Decreased Total NAD Content and a Possible Secondary Cause of Hemolysis

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Erythrocytes from individuals with pyruvate kinase (PK)-deficiency have approximately half the total (oxidized and reduced) nicotinamide adenine dinucleotide (NAD) of normal erythrocytes. In order to elucidate the mechanism(s) for the decrease in total NAD, we examined NAD synthesis in intact erythrocytes. It is demonstrated that NAD synthesis is impaired in PK-deficient erythrocytes to a degree that is dependent on the PK activity and adenosine 5'-triphosphate (ATP) concentration of these cells. After incubation in the presence of fluoride, which simulates the characteristics of PK deficiency by inhibiting enolase, normal erythrocytes had impaired NAD synthesis and decreased ATP concentrations. Fluoride did not inhibit NAD synthesis in a hemolysate system that is not dependent on glycolysis for ATP generation. These data suggest that fluoride does not inhibit the enzymes of NAD synthesis and that impairment of NAD synthesis by fluoride is mediated by decreased ATP formation. Thus, it is concluded that impaired NAD synthesis in PK-deficient erythrocytes is caused by decreased ATP formation due to the PK deficiency. Since the rate of glycolysis is limited by the availability of NAD⁺, it is suggested that impaired NAD synthesis causes further ATP depletion and thereby may enhance hemolysis in PK-deficient erythrocytes.

MATERIALS AND METHODS

Materials

d-Glucose was purchased from J.T. Baker Chemical Co, Phillipsburg, NJ. All other reagents were purchased from Sigma Chemical Co, St Louis. The potassium salt of fluoride was used throughout this investigation.

Methods

Isolation of erythrocytes. After obtaining informed consent, blood was obtained by routine venipuncture from PK-deficient erythrocytes.
conditions were used: complete system (closed circles), without glutamine. and 5.0 mmol/L inorganic phosphate; other conditions were used (5.0 mmol/L glucose, 1.0 mmol/L inorganic phosphate; other conditions are the same as for the optimal system). The following incubation conditions were used (see Materials and Methods). (B) Physiological conditions were used (5.0 mmol/L glucose, 1.0 mmol/L glutamine, and 5.0 mmol/L inorganic phosphate; other conditions are the same as for the optimal system). The following incubation conditions were used: complete system (closed circles), without glutamine (open circles).

Fig 1. NAD biosynthesis in human erythrocytes. The reactions shown are catalyzed by nicotinic acid phosphoribosyltransferase (EC 2.4.2.11), reaction a; nicotinic acid adenine dinucleotide pyrophosphorylase (EC 2.7.7.18), reaction b; and NAD synthetase (EC 6.3.5.1), reaction c. AMP, adenosine 5'-monophosphate; PP, inorganic pyrophosphate.

Individuals and anticoagulated with heparin (15 U/mL whole blood). Blood from normal subjects and from individuals with hemolytic anemia of unknown etiology served as appropriate controls. The separation and washing of erythrocytes have been described previously.13 Erythrocytes were used immediately after isolation.

Preparation of hemolysates. Hemolysates used to determine PK activity were prepared by using a freeze-thaw method.12 Hemolysates used to measure NAD synthesis were prepared by using osmotic lysis of intact cells without freeze-thawing since the latter manipulation markedly decreases the conversion of nicotinic acid to the NAD precursor nicotinic acid mononucleotide14 (Fig 1, reaction a). Suspensions of erythrocytes were centrifuged at 3,000 g for 10 minutes. Two volumes of a solution containing 2.7 mmol/L EDTA, pH 7.0, and 0.70 mmol/L 2-mercaptoethanol were added to the packed erythrocytes and mixed gently. Examination under a light microscope revealed that more than 99.9% of the erythrocytes were lysed after this treatment. This hemolysate was used immediately in the NAD synthesis assay.

NAD synthesis assay. Conditions for the NAD synthesis assay in intact erythrocytes were a modification of the system of Preiss and Handler.16 The incubation mixture contained 40 μmol potassium phosphate, pH 7.4; 75 μmol KCl; 75 μmol NaCl; 30 μmol D-glucose; 1.0 μmol nicotinic acid; 5.0 μmol L-glutamine; and intact erythrocytes equivalent to 42 mg hemoglobin (Hb) in a total volume of 1.00 mL. The assay was started by adding the appropriate volume of erythrocyte suspension, and the complete incubation mixture was maintained at 37°C. Conditions for the NAD synthesis assay in hemolysate were developed by modifying the previous incubation mixture. Glucose and inorganic phosphate were replaced with PRPP so that NAD synthesis would be independent of glycolysis. NaCl was eliminated since the maintenance of isotonic conditions was no longer necessary, and MgCl₂ was added since the endogenous concentration of magnesium is, presumably, decreased after cell lysis due to dilution of the cell contents. Thus, the incubation mixture for NAD synthesis in hemolysate contained 10 μmol Tris-HCl, pH 7.4; 5.0 μmol KCl; 1.0 μmol nicotinic acid; 15 μmol MgCl₂; 5.0 μmol PRPP; 5.0 μmol ATP; 5.0 μmol L-glutamine; and hemolysate equivalent to 42 mg Hb in a total volume of 1.00 mL. The assay was started by adding the appropriate volume of hemolysate, and the complete incubation mixture was maintained at 37°C.

NAD synthesis in both the intact erythrocyte and hemolysate incubation mixtures was determined by measuring the accumulation of the total (oxidized and reduced) NAD concentration after various times of incubation. Thus, at the times indicated, a 0.20-mL aliquot was withdrawn, maintained in a boiling water bath for 60 seconds, and then cooled to 0°C. The heat-treated aliquot was centrifuged to remove cell debris and denatured proteins. To minimize interference by compounds present in the incubation mixture, the supernatant was diluted tenfold before determination of the total NAD concentration with the sensitive cycling method of Bernofsky and Swan.15 The total NAD concentration, instead of oxidized NAD (NAD⁺), was measured since NAD⁺ can readily be converted to its reduced form (NADH) following synthesis.

ATP formation from PRPP. Conditions for ATP formation from PRPP and endogenous AMP and inorganic phosphate were identical to those used to measure NAD synthesis in the hemolysate.
system (see earlier) except that ATP was omitted from the reaction mixture. When ATP formation from PRPP was measured in the presence of exogenous AMP and inorganic phosphate, the aforementioned reaction mixture was supplemented with 1.0 mmol/L AMP and 20 mmol/L potassium phosphate (pH 7.4). At the indicated times, aliquots were withdrawn and heat-treated as described earlier. Following centrifugation, the supernatant was used to quantify ATP as described by Beutler.16

The rate of ATP formation was also measured by using a continuous spectrophotometric assay that contained 10 μmol Tris-HCl, pH 7.4; 5.0 μmol KCl; 1.0 μmol nicotinic acid; 15 μmol MgCl2; 5.0 μmol PRPP; 5.0 μmol L-glutamate; 0.40 μmol NADP; 1.0 μmol D-glucose; 1.2 IU glucose-6-phosphate dehydrogenase; 8.0 IU hexokinase; and hemolysate equivalent to 1.7 mg Hb in a total volume of 1.00 mL. The reaction mixture was preincubated at 37°C and then started by adding the appropriate volume of PRPP. The absorbance was monitored at a wavelength of 340 nm by using a Gilford model 252 spectrophotometer (Gilford, Ciba-Corning Diagnostics, Oberlin, OH) with a slit width of 0.80 mm. The relatively wide slit width was required because the amount of hemolysate necessary for the reaction resulted in a relatively high initial absorbance. However, because the absorbance band of NADPH is fairly wide, the millimolar extinction coefficient of NADPH remains 6.22 at a slit width of up to 1.0 mm.16

Other methods. PK activity was determined as described by Tanaka.17 The whole blood ATP content was determined spectrophotometrically10 by using neutralized perchloric acid extracts.14 This method was also used to measure the ATP concentration in intact erythrocytes during incubation with fluoride. The Hb concentration was determined by using the cyanmethemoglobin method.16 Data were expressed as the mean ± 1 SD.

RESULTS

Intact human erythrocytes are capable of NAD synthesis when incubated in the presence of glucose, inorganic phosphate, glutamine, and nicotinic acid.10,19 We have found that normal erythrocytes increased their original total NAD content five to 15 times after incubation with the latter compounds for 20 hours (Fig 2A). Relatively little total NAD was accumulated in the absence of glutamine from the incubation mixture (Fig 2A). Since glutamine is essential for NAD synthesis in human erythrocytes,11 this suggests that the total NAD accumulation is due to NAD synthesis. To a lesser extent, the total NAD also accumulated when the concentrations of glucose, glutamine, and inorganic phosphate in the incubation mixture were reduced to simulate more physiological conditions (Fig 2B). This suggests that NAD synthesis in normal mature RBC is physiologically significant.

The quantity of total NAD accumulated after 20 hours of incubation in erythrocytes from eight normal subjects was 1.35 ± 0.24 μmol/g Hb (Table 1). RBC from five patients with hemolytic anemia of unknown etiology (9.0% to 15% reticulocytes) accumulated 1.14 ± 0.14 μmol NAD/g Hb. In contrast, erythrocytes from three PK-deficient patients accumulated substantially less total NAD than erythrocytes from normal subjects (Table 1). NAD synthesis in PK-deficient erythrocytes correlated positively with PK activity and the level of intracellular ATP. Erythrocytes from PK-deficient patient 3, who had the highest PK activity and ATP content, accumulated 1.04 μmol total NAD/g Hb (Table 1). In contrast, erythrocytes from PK-deficient patient 1, who had the lowest ATP content, accumulated only 0.53 μmol total NAD/g Hb (Table 1). Since this patient had undergone splenectomy (Table 1), presumably a greater proportion of more defective erythrocytes was in circulation.

Since the capacity of PK-deficient erythrocytes to synthesize NAD correlated with their ATP content, we examined the total NAD accumulation in normal erythrocytes using fluoride to inhibit enolase26 and reduce ATP formation. Incubation of intact normal erythrocytes with fluoride under conditions that yield optimal NAD synthesis resulted in ATP depletion to a degree that was dependent on the concentration of fluoride added to the incubation mixture (Fig 3). Hence, as little as 0.1 mmol/L fluoride led to a 28% decrease in the ATP content after six hours of incubation (Fig 3). A fluoride concentration of 1.0 mmol/L was sufficient to cause a 34% decrease in the ATP concentration after only three hours of incubation (Fig 3). The addition of fluoride to the incubation mixture resulted in marked inhibition of NAD synthesis in normal intact erythrocytes. A fluoride concentration of only 0.10 mmol/L caused a 17% inhibition of total NAD accumulation (Fig 4). Higher concentrations of fluoride were more effective in impairing NAD synthesis, with 1.0 mmol/L fluoride causing full inhibition of total NAD accumulation (Fig 4).

The impairment of NAD synthesis by fluoride is most likely due to decreased ATP regeneration. However, since it is not known what effect fluoride has on the enzymes of NAD synthesis, we determined the effect of fluoride on these enzymes by measuring total NAD accumulation in a hemolysate system that is not dependent on glycolysis (and enolase).

Since the present experiment is the first known demonstration of NAD synthesis in a human hemolysate system, the
Fig 3. The effect of fluoride on the ATP concentration of intact normal erythrocytes under optimal conditions for NAD synthesis. The following incubation conditions were used: complete system (circles), with 0.10 mmol/L fluoride (triangles), with 1.0 mmol/L fluoride (squares).

effect of omissions of various components of the incubation mixture was examined. There was no total NAD accumulation in the absence of nicotinic acid, MgCl₂, glutamine, or PRPP after 20 hours of incubation (Table 2). In contrast, the absence of ATP resulted only in a 32% decrease in the total NAD accumulation after 20 hours of incubation (Table 2). However, there was no total NAD accumulation when both ATP and PRPP were excluded from the incubation mixture (Table 2). This suggests that the substantial accumulation of total NAD observed in the absence of added ATP may be due to ATP regeneration from PRPP and endogenous AMP. Previous studies have shown that PRPP synthetase (EC 2.7.6.1), the enzyme responsible for PRPP synthesis from ATP and ribose-5-phosphate, can catalyze the reverse reaction nearly as well as the forward reaction. To determine whether ATP was being regenerated from PRPP under our experimental conditions, we measured ATP formation under the same conditions that yield optimal NAD synthesis in our hemolysate system. To ensure that the presence of the NAD precursors does not interfere with the reverse PRPP synthetase reaction, we first examined ATP formation from PRPP when AMP and inorganic phosphate were exogenously supplied to the reaction mixture. The results indicate that ATP accumulated in a time-dependent manner in the presence of PRPP (Fig 5A). No ATP was found to accumulate when PRPP was absent from the reaction mixture (Fig 5A). This suggests that the NAD precursors present in the reaction mixture do not interfere with ATP formation from PRPP.

Subsequently, we examined ATP formation from PRPP using the hemolysate’s endogenous AMP and inorganic phosphate. Under these conditions, no accumulation of ATP was observed in the presence of PRPP (Fig 5B). However, in the absence of PRPP, the ATP content decreased rapidly (Fig 5B). The lack of ATP accumulation in this system suggested that the rate of ATP formation from PRPP and endogenous AMP and inorganic phosphate was not rapid enough to overcome the rate of ATP utilization. To confirm this hypothesis, we devised a continuous spectrophotometric system in which the formation of ATP results in the production of NADPH and a concomitant increase in the absorbance at 340 nm. In this system, the ATP that is formed is “trapped” by the detection system before it can be utilized for NAD synthesis and/or other processes. Under these conditions, ATP was formed readily from endogenous AMP and inorganic phosphate in the presence of PRPP and hemolysate (Fig 5C). It is noteworthy that a significant rate of ATP formation (4.6 µmol/h · g Hb at steady state) was obtained with only 1/25th the hemolysate (1.7 mg Hb) used for the NAD synthesis assay (Fig 5C). No significant ATP formation was observed in the absence of either PRPP or hemolysate (Fig 5C). Furthermore, the addition of exogenous AMP and inorganic phosphate to this system yielded a more rapid rate of ATP formation (Fig 5C) that is consistent with the rapid ATP accumulation of Fig 5A. These data confirm that ATP is formed from PRPP and endogenous AMP and inorganic phosphate under optimal conditions for

Table 2. The Effect of Omissions of Various Components of the Incubation Mixture on NAD Synthesis in Hemolysate

<table>
<thead>
<tr>
<th>Contents of Incubation Mixture</th>
<th>Total NAD Accumulated in 20 Hours (µmol/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.361</td>
</tr>
<tr>
<td>Without nicotinic acid</td>
<td>0</td>
</tr>
<tr>
<td>Without MgCl₂</td>
<td>0</td>
</tr>
<tr>
<td>Without glutamine</td>
<td>0</td>
</tr>
<tr>
<td>Without PRPP</td>
<td>0</td>
</tr>
<tr>
<td>Without ATP</td>
<td>0.247</td>
</tr>
<tr>
<td>Without ATP and PRPP</td>
<td>0</td>
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</tbody>
</table>

Fig 4. The effect of fluoride on NAD synthesis in intact normal erythrocytes under optimal conditions. The following incubation conditions were used: complete system (closed circles), without glutamine (open circles), with 0.10 mmol/L fluoride (closed triangles), with 0.50 mmol/L fluoride (open triangles), with 1.0 mmol/L fluoride (squares).
NAD synthesis in hemolsate. Thus, the substantial quantities of total NAD accumulated in the absence of ATP and in the presence of PRPP (Table 2) is due to the regeneration of ATP from PRPP.

In the hemolsate-NAD synthesis system, there was an initial decrease in the total NAD concentration within the first two hours of incubation (Fig 6). Subsequently, total NAD accumulated at a linear rate up to 20 hours of incubation (Fig 6). The addition of up to 1.0 mmol/L fluoride to the incubation mixture did not affect total NAD accumulation in the hemolsate system (Fig 6). The initial decrease in total NAD concentration that is observed in this system (Fig 6) may be caused by the exposure of the intraerythrocytic NAD pool to the extracellular membrane-bound NADase after red cell lysis. The lack of a decrease in the total NAD content when intact erythrocytes are used (Figs 2A and 2B) is consistent with this hypothesis since intraerythrocytic NAD is not exposed to NADase under these conditions.

**DISCUSSION**

We have shown that intact PK-deficient erythrocytes have impaired NAD synthesis since they accumulated less total NAD than normal erythrocytes when incubated with precursors essential for NAD biosynthesis (Table 1). Impaired NAD synthesis in PK-deficient red cells is not due to the higher proportion of reticulocytes since high reticulocyte red cell samples without PK deficiency did not demonstrate significantly impaired NAD synthesis. The positive correlation between NAD synthesis, PK activity, and the level of...
fluoride (closed triangles), with 0.50 mmol/L fluoride (open triangles), with 1.0 mmol/L fluoride (squares).

Fig 6. The effect of fluoride on NAD synthesis in hemolysate from normal erythrocytes. The following incubation conditions were used: complete system (closed circles), with 0.10 mmol/L fluoride (closed triangles), with 0.50 mmol/L fluoride (open triangles), with 1.0 mmol/L fluoride (squares).

Intraerythrocytic ATP suggested that impaired NAD synthesis in PK-deficient erythrocytes may be caused by decreased ATP regeneration due to low PK activity. To provide further evidence for this hypothesis, we measured total NAD accumulation in normal erythrocytes in the presence of fluoride. This ion is an inhibitor of enolase and has been used previously to simulate the characteristics of PK deficiency. The decrease in the ATP concentration with time at all fluoride concentrations used (Fig 3) suggests that fluoride was inhibiting enolase (and glycolysis) and, presumably, simulating PK deficiency under the conditions used.

We have found that fluoride is a potent inhibitor of NAD synthesis in intact erythrocytes (Fig 4) even at concentrations that cause only partial depletion of ATP (Fig 3). The sensitivity of NAD synthesis to inhibition by fluoride may be due to the requirement of several ATP molecules for the synthesis of one NAD molecule (Fig 1). The inhibition of NAD synthesis in intact erythrocytes by fluoride cannot be due to inhibition of PRPP synthetase since fluoride concentrations as high as 50 mmol/L do not affect PRPP synthetase activity in crude hemolysate. To determine whether fluoride interfered with the activity of the enzymes of NAD biosynthesis, we measured the total NAD accumulation in hemolysate by substituting PRPP for glucose and inorganic phosphate. Under these conditions, NAD synthesis will not be dependent on glycolysis and hence enolase to provide ATP for PRPP formation. There was a substantial accumulation of total NAD in this hemolysate system (Table 2 and Fig 6). Since there was no accumulation of total NAD when components essential for NAD synthesis such as nicotinic acid, glutamine, MgCl₂, or PRPP were excluded (Table 2), it is suggested that the total NAD accumulation in the hemolysate system was due to NAD synthesis. The substantial accumulation of total NAD observed in the absence of added ATP is due to ATP regeneration from PRPP and endogenous AMP since no total NAD accumulation is observed in the absence of both ATP and PRPP (Table 2) and since ATP formation from PRPP and endogenous AMP can be readily demonstrated (Fig 5C). When NAD synthesis was measured in the hemolysate system and glycolysis and enolase were bypassed, fluoride had no significant effect on total NAD accumulation (Fig 6). This suggests that fluoride did not inhibit the enzymes of NAD biosynthesis in hemolysate.

The lack of inhibition of the NAD biosynthetic enzymes and PRPP synthetase by fluoride in hemolysate suggests that impairment of NAD synthesis in intact normal erythrocytes in the presence of fluoride is caused by decreased ATP formation. These data support the hypothesis that impaired NAD synthesis in intact PK-deficient erythrocytes is caused by decreased ATP regeneration due to the PK deficiency itself. Furthermore, our data suggest that impaired NAD synthesis is a primary cause of the decreased total NAD concentration in PK-deficient erythrocytes. Previous studies from this laboratory have shown that PK-deficient erythrocytes have decreased PRPP synthetase subunit aggregation and therefore a less active enzyme in vivo. This is caused by the decreased ATP and increased 2,3-diphosphoglycerate concentrations in these erythrocytes. Thus, we have suggested that impaired NAD synthesis may also be mediated by the decreased in vivo activity of PRPP synthetase.

Impaired NAD synthesis in PK-deficient erythrocytes is significant because the rate of the G3PD reaction and hence glycolysis is limited by the NAD⁺ content of the erythrocyte. Thus, impaired NAD synthesis may cause further ATP depletion and may be a secondary cause of hemolysis in PK-deficient erythrocytes.

ACKNOWLEDGMENT

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REFERENCES

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