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$NADP^+$ AND NADPH IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT ERYTHROCYTES UNDER OXIDATIVE STIMULATION

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SUMMARY

A mild oxidative stimulation of the hexose monophosphate pathway of human glucose-6-phosphate dehydrogenase (EC 1.1.1.49)-deficient erythrocytes (Mediterranean variant) causes a significant drop in NADPH.

These results, other than to confirm that glucose-6-phosphate dehydrogenase deficiency is a product deficiency disorder, demonstrate that under oxidative stimulation glutathione reductase may become functionally impaired and GSSG cannot be reduced at a sufficient rate.

INTRODUCTION

Several studies indicate that the functional impairment in human erythrocytes deficient in glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49) is a decreased capacity to maintain GSH at normal levels, especially in the presence of oxidizing agents [1, 2]. Conversely, GSSG is returned to GSH by glutathione reductase, an enzyme which requires NADPH. Since glucose-6-phosphate dehydrogenase and its tandem enzyme, 6-phosphogluconate dehydrogenase, are the principal source of NADPH in the human erythrocytes, the defect in the glucose-6-phosphate dehydrogenase-deficient cell should be the failure to generate NADPH at a rate sufficient to meet these needs [3, 4]. Previous studies have demonstrated that, in steady-state conditions, Mediterranean and A^-, glucose-6-phosphate dehydrogenase-deficient cells have a low NADPH/NADP ratio [5]. We here report the intracellular concentration of total NADP ($NADP^+ + NADPH$) and NADPH, during in vitro oxidative stimulation of the hexose monophosphate pathway in normal and Mediterranean glucose-6-phosphate dehydrogenase-deficient erythrocytes.

Abbreviation: TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

MATERIALS AND METHODS

Blood samples were obtained from eight normal and seven Mediterranean, glucose-6-phosphate dehydrogenase-deficient men with no apparent haematological disease and normal packed cell volume. Screening for glucose-6-phosphate dehydrogenase deficiency was performed by the brilliant cresyl blue method [6] and the assay for the enzyme was carried out spectrophotometrically by the measurement of the rate of formation of NADPH in the presence of the enzyme, glucose 6-phosphate and $NADP^+$ [6]. Defibrinated blood was centrifuged at $1000 \times g$ per 15 min, and serum and buffy coat were removed. The erythrocytes were then mixed with five volumes of 0.15 M NaCl and centrifuged again. Supernatant fluid was discarded and the erythrocytes were suspended in equal volume of Krebs-Ringer buffer [7] which contained glucose at a final concentration of 5 mM, and *N*-tris-(hydroxymethyl) methyl-2-aminoethanesulphonic acid (TES) (sodium salt, pH 7.4) at a final concentration of 20 mM [3].

The incubation mixture consisted of 1.0 ml of the erythrocyte suspension and 1.3 ml of the Krebs-Ringer TES glucose buffer with and without α-naphthol at a final concentration of 0.08 mM. The incubation was performed in scintillation flasks in a Dubnoff metabolic shaker at 37 °C for 6 h at 120 oscillations/min. At the end of the incubation total NADP ($NADP^+ + NADPH$) and NADPH were measured as described before [5]. Haematocrits were performed in order to determine true cell volumes and the values of the nucleotides were expressed as μmol/l of erythrocytes. The method is based on the extraction procedure with 0.04 M NaOH containing 0.5 mM cysteine according to Burch et al. [8] and the cycling method of Lowry and Passonneau [9]. α-naphthol, at the concentration in the incubation mixtures, was present in the blanks, as well as in the $NADP^+$ and NADPH standards. Incubation and nucleotides determinations were performed in duplicate.

RESULTS AND DISCUSSION

6 h after the incubation of normal erythrocytes at 37 °C in normal conditions, almost all NADP was in the reduced form, with an $NADPH/(NADP^+ + NADPH)$ ratio of 0.9. The incubation in the presence of α-naphthol did not alter the ratio as well as the reduced and total amount of the nucleotide (Table I).

Behaviour of Mediterranean, glucose-6-phosphate dehydrogenase-deficient erythrocytes, was different. In the steady state these cells have most of the nucleotide in the oxidized form (Table I). A small increase in the oxidative rate induced by the oxidizing agent caused a significant reduction in the $NADPH/(NADP^+ + NADPH)$ ratio and in the concentration of NADPH (Table I).

The mean total amount of NADP ($NADP^+ + NADPH$) in Mediterranean glucose-6-phosphate dehydrogenase-deficient erythrocytes, incubated in the presence of the naphthol, increased with respect to the cells incubated without it (Table I). The NADPH values observed in incubated erythrocytes, both normal and glucose-6-phosphate dehydrogenase-deficient, were slightly lower compared to those observed in fresh cells [5]. The phenomenon was also noticed by Omachi et al. [10] on normal erythrocytes.

Previous in vitro studies with α-naphthol, a metabolite of naphthalene known

TABLE I

NADPH AND $NADP^+$+NADPH CONCENTRATIONS IN NORMAL AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT ERYTHROCYTES AFTER SIX HOURS INCUBATION AT 37 °C

Values are means ±S.D. and are expressed as μmol/l of erythrocytes. Number of experiments is given in parenthesis. P values for the significance between paired observations: normal erythrocytes, $*P > 0.2$, $**P > 0.4$, $***P > 0.4$; glucose-6-phosphate dehydrogenase-deficient erythrocytes, $*P < 0.0005$, $**P < 0.01$, $***P < 0.0005$.

Subjects	α-Naphthol		
	0 μM		
	NADPH*	$NADP^+$ +NADPH**	NADPH*** (NADP⁺+NADPH)
Normal (8)	28.59±4.43	31.30±4.30	0.9138±0.0534
Glucose-6-phosphate dehydrogenase-deficient (7)	12.39±3.24	63.97±4.73	0.1943±0.0447

Subjects	α-Naphthol		
	80 μM		
	NADPH*	$NADP^+$ +NADPH**	NADPH*** (NADP⁺+NADPH)
Normal (8)	28.16±5.17	31.10±5.17	0.9050±0.0447
Glucose-6-phosphate dehydrogenase-deficient (7)	4.49±2.28	67.59±6.77	0.0686±0.0363

to cause haemolysis in glucose-6-phosphate dehydrogenase-deficient subjects when accidentally ingested [11], at concentrations probably occurring in vivo, caused a severe depletion of GSH, without stimulation of the hexose monophosphate pathway in Mediterranean glucose-6-phosphate dehydrogenase-deficient erythrocytes [3]. This fact could not be expected taking in consideration the substrate availability or the properties of the enzyme in a cell-free system. Since the hexose monophosphate pathway in Mediterranean glucose-6-phosphate dehydrogenase-deficient erythrocytes is operating at its maximum intracellular rate, it has been demonstrated that glucose-6-phosphate dehydrogenase is under an unexplained intracellular restraint [3]. On this basis it was possible to explain the low NADPH/($NADP^+$+NADPH) ratio observed in the steady state [5] and to predict, taking into consideration the known K_m and K_i values for NADPH, the possible variations in the ratio of the nucleotides under different rates of oxidation of NADPH. A slight increase in the rate of oxidation observed in this study is responsible for the significant drop in the NADPH/($NADP^+$+NADPH) ratio.

These findings indicate that the availability of NADPH in glucose-6-phosphate dehydrogenase deficiency is the limiting factor in the mechanism of detoxification of hydrogen peroxide produced by the erythrocytes [12].

Furthermore, glutathione reductase is the enzyme preposed to the reduction of GSSG in the presence of NADPH. Since several authors have reported for this

enzyme a K_m for NADPH varying between 10 and 16 μM [13, 14], these values are considerably higher in respect to the NADPH concentrations observed in deficient erythrocytes in this study. Probably, in in vivo conditions, the NADPH values could be lower, owing to a longer period of exposure of the cells to the oxidizing agent.

The consequence is that the activity of glutathione reductase may become functionally impaired, and GSSG cannot be reduced at a sufficient rate. On the other hand higher glutathione reductase activity has been described in glucose-6-phosphate dehydrogenase-deficient erythrocytes [15, 16] without any apparent explanation. It may now be interpreted as a compensatory mechanism to the low NADPH values present in these cells. Normal erythrocytes, with NADPH concentrations of the order of 30 μM, which are considerably higher in respect to the K_m values for glutathione reductase, have a lower enzyme activity. Glucose-6-phosphate dehydrogenase-deficient cells also have a higher total amount of NADP in respect to normal erythrocytes (Table I). A possible explanation is that this may represent a cellular adaptation to the low concentrations of NADPH. The phenomenon seems to be confirmed by the fact that, even during a short period of increased rate of NADPH oxidation, glucose-6-phosphate dehydrogenase-deficient erythrocytes tend to increase significantly (Table I) the total amount of nucleotide, to reduce the effect of oxidation of NADPH. Normal erythrocytes, with no variations in the amount of NADPH, have an unchanged concentration of total nucleotide at the end of the incubation.

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