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$NADP^+$ reduction by human lymphocytes

A. KLEIN, A. W.-L. CHAN, B. U. CAPLAN & A. MALKIN
Department of Clinical Biochemistry, Sunnybrook Medical Centre, University of Toronto, Toronto, Ontario, Canada

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SUMMARY

The hexose monophosphate shunt (HMPS) is known to be responsible for the reduction of $NADP^+$ by lymphocytes. We tried to find other enzymatic systems that might provide the lymphocytes with NADPH. By measuring the absorbance at 340 nm we noted that the addition of $NADP^+$ to a preparation of disrupted lymphocytes resulted in the formation of NADPH at a rate of 4 nmol/10^6 cells per min. This phenomenon could not be changed by negative feedback inhibition of HMPS, and could not be attributed to the low concentration of glucose, glucose-6-phosphate (G-6-P) and isocitrate found in the cell preparation ($NADP^+$-dependent isocitrate dehydrogenase in addition to HMPS $NADP^+$ reducing enzymes was found to be present in lymphocytes). Because of the activity of a $NADP^+$-dependent lactate dehydrogenase, pyruvate oxidized the NADPH as it was being formed. Here we demonstrate the presence of an unknown $NADP^+$ reducer in lymphocytes which seems to play an additional role to HMPS in $NADP^+$ reduction by lymphocytes. $NADP^+$-dependent lactate dehydrogenase may play a role in regulating the $NADP^+$/NADPH ratio.

Keywords $NADP^+$ NADPH lymphocytes hexose monophosphate shunt pyruvate

INTRODUCTION

NADPH is essential for protein, lipid and DNA synthesis. The hexose monophosphate shunt (HMPS) is known to be the source of this reduced nucleotide in lymphocytes (McHaffie & Wang, 1967; Sagone, LoBuglio & Balcerzak, 1974; Tsan *et al.*, 1976; Gartler, Hornung & Motulsky, 1981). An increase in the activity of the shunt is found in mitogen-stimulated lymphocytes (McHaffie & Wang, 1967; Sagone *et al.*, 1974; Tsan *et al.*, 1976) and is also characteristic of the 'respiratory burst' found with phagocytosing cells (Babior, 1978; Chaundry, Santinga & Gabig, 1982; Suzuki & Kakinuma, 1983). The aims of the present study were to see whether alternative pathways to HMPS are involved in $NADP^+$ reduction and whether $NADP^+$-dependent lactate dehydrogenase is involved in NADPH regulation (Suzuki & Kakinuma, 1983).

MATERIALS AND METHODS

Lymphocytes were prepared from peripheral blood of five healthy donors. They were isolated by centrifugation at 400 ***g*** using the Ficol–Isopac method, washed twice, and suspended in phosphate-buffered saline (PBS) (pH 7·2) containing penicillin and streptomycin, 100 U each. To ensure $NADP^+$ penetration (Suzuki & Kakinuma, 1983) the lymphocytes were disrupted by freezing and thawing three times, using an acetone–dry ice bath. After homogenization with a glass homogenizer cells were divided into two groups; one group did not undergo any further treatment, and the other was dialysed against HBSS for 24 h at 4°C with two solution changes. The dialysis procedure was utilized to remove potential $NADP^+$ reducers.

For reconstitution experiments, a portion of the disrupted cells were dialysed against water. The dialysate was lyophilized. The dry remnant was added to dialysed cells in HBSS and $NADP^+$ reduction was measured. Disrupted cells from the first group (undialysed) were kept for 24 h at 4°C as control for the second group (dialysed).

Each preparation of lymphocytes was divided into aliquots containing an average of 5×10^7 disrupted cells in 0·7 ml of suspension. To measure the conversion of $NADP^+$ to NADPH, $NADP^+$ was added to the various lymphocyte preparations at 20°C. After incubation for 2 min, the samples were centrifuged at 12000 ***g*** for 5 min at 4°C. The supernatant was transferred to spectrometer cuvettes and the absorbance read at 340 nm using a Pye Unicam spectrophotometer.

The effect of the following substances on reduction of $NADP^+$ was measured: glucose-1-P; glucose with or without ATP; glucose-six-phosphate (G-6-P); malate; isocitrate; lactate; pyruvate; and glutamate.

Correspondence: Dr A. Klein, Department of Clinical Biochemistry, Sunnybrook Medical Centre, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5, Canada.

In a separate experiment ribulose-5-phosphate and gluconate-6-phosphate were added for their negative feedback effect on HMPS $NADP^+$ reducing enzymes.

In another experiment, the concentrations of G-6-P and isocitrate in lymphocytes were measured according to the methods of Lang & Michol (1974), and Siebert (1974). Glucose concentration was measured by the glucose oxidase method using oxygen electrode (Astra system, Beckman Instruments, Mountain View, CA). The experiments were repeated five times.

Statistical analysis included one-way analysis of variance followed by Duncan's multiple range test.

RESULTS

Figure 1 shows the reduction of $NADP^+$ obtained from disrupted lymphocytes after 2 min of incubation at 20°C. The incubation temperature of 20°C was chosen as a result of preliminary studies showing that the reaction was too fast, at 37°C. Monitoring the pH showed no change at the end of the reaction. Heating the preparation at 80°C for 5 min abolished its capability to reduce $NADP^+$. The level of reduction obtained with 0·4 μmol $NADP^+$ (which was equal to the absorbance obtained with 0·4 μmol NADPH) could not be increased even by raising the amount of $NADP^+$ to 4 μmol. In order to find out whether there is a correlation between these findings and the participation of HMPS in $NADP^+$ reduction by lymphocytes, the G-6-P and glucose contents in 5×7^{10} lymphocytes were measured. The amount of G-6-P was found to be 13 ± 2 nmol/5×10^7 cells, and that of glucose 21 ± 3 nmol/5×10^7 cells.

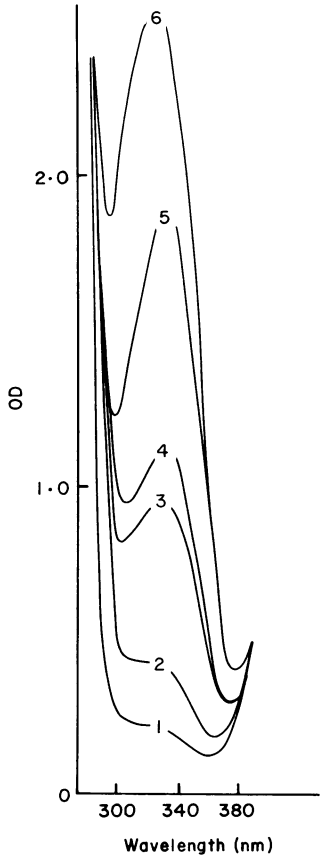

Fig. 1. The reduction of incremental concentrations of $NADP^+$ and NAD^+ obtained by disrupted lymphocytes (either fresh or stored for 24 h at 4°C). Incubation time was 2 min at 20°C; 5×10^7 cells immersed in 0·7 ml PBS. 1, control (lymphocytes with no nucleotides added); 2, NAD^+ (0·4 μmol) + lymphocytes; 3, NAD^+ (0·8 μmol) + lymphocytes; 4, NAD^+ (1·2 μmol) + lymphocytes; 5, $NADP^+$ (0·2 μmol) + lymphocytes; 6, $NADP^+$ (0·4 μmol) + lymphocytes. The results described by curve no. 6 remained unaffected after the addition of ribulose-5 and gluconate-6-phosphate (each 2 μmol) to the preparation before the addition of the nucleotide.

In another experiment the HMPS NADP reducing enzymes, namely G-6-P dehydrogenase and phosphogluconate dehydrogenase were first inhibited by their products, i.e. 6-phosphogluconate (2 μmol) and D-ribulose-5-phosphate (2 μmol). They were added to the lymphocyte preparation (0·7 ml) before the addition of $NADP^+$ (0·4 μmol) and before the start of the incubation. 6-Phosphogluconate and D-ribulose-5-phosphate failed to change the $NADP^+$ reduction demonstrated in Fig. 1.

On comparing the effect of incremental concentrations of $NADP^+$ and NAD^+ on their own reduction in disrupted lymphocytes, a higher rate of reduction was obtained with $NADP^+$. The 340 nm absorbance peak obtained with 0·4 μmol $NADP^+$ was 6·1 times higher than the one obtained with the same concentration of NAD^+ (Fig. 1).

The time-course demonstrated in Fig. 2 shows that a complete reduction of $NADP^+$ was accomplished 1·5 min after the start of incubation.

Since the foregoing results suggested the presence of $NADP^+$ reducers in lymphocytes, dialyzed disrupted cells were then used. The marked increase in $NADP^+$ reduction found with undialysed disrupted cells seen in Fig. 1 was no longer observed with dialysed disrupted cells after the addition of $NADP^+$. This was probably a result of the loss of a reducer of $NADP^+$ because of dialysed disrupted cells with reconstituted dialysate recovered the capacity of the cells to reduce $NADP^+$ (Fig. 3).

In dialysed disrupted cells 1 μmol G-6-P and isocitrate were capable of reducing $NADP^+$ (Fig. 4). However, this reduction was lower (3·7 and 4·3 times, respectively, than that obtained with the undialysed disrupted cells) (Fig. 1). No G-1-P, malate, glutamate, glucose (with and without ATP), lactate nor glutathione were active in this regard (Fig. 3). It should be emphasized that the amount of glucose, isocitrate and G-6-P found in 5×10^7 undialysed cells was extremely low.

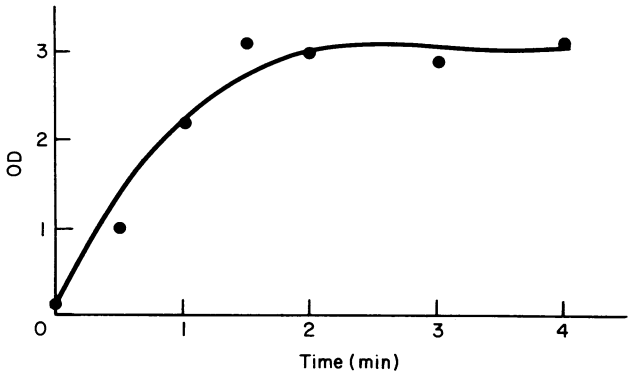

Fig. 2. Time-course of 0·4 μmol $NADP^+$ reduction obtained by disrupted lymphocytes, as described in Fig. 1.

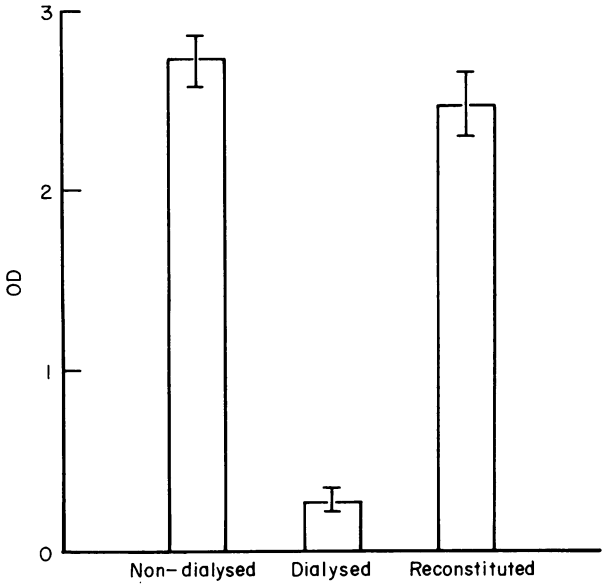

Fig. 3. Reduction of $NADP^+$ (0·4 μmol) by disrupted dialysed lymphocytes before and after reconstitution with the dialysate. The preparations were incubated at 20°C for 2 min in 0·7 ml HBSS.

Figure 5 demonstrates the negative effect of pyruvate on the reduction of $NADP^+$ obtained by undialysed disrupted lymphocytes. In order to find out whether this effect is a result of either enzymatic inhibition or the presence of $NADP^+$-dependent lactate dehydrogenase we added pyruvate after the formation of the NADPH peak, and after the addition of NADPH itself to a dialysed lymphocyte preparation. In both cases the NADPH peaks disappeared and pyruvate was converted to lactate.

Heating the preparation at 80°C for 5 min abolished the capability of it to oxidize NADPH in the presence of pyruvate. The time-course of the oxidation of NADPH due to addition of pyruvate is shown in Fig. 6.

DISCUSSION

G-6-P dehydrogenase, as a part of HMPS is well known to be an important source of reduced $NADP^+$ in lymphocytes (McHaffie & Wang, 1967; Sagone *et al.*, 1974; Tsan *et al.*, 1976; Gartler *et al.*, 1981). Our attempt to find other dehydrogenases which can supply the cells with NADPH, revealed that, only $NADP^+$ dependent isocitrate dehydrogenase is involved in $NADP^+$ reduction. Figure 3 shows that this low activity obtained in the presence of either G-6-P or isocitrate was not due to loss of enzyme activity during dialysis.

The fast reduction of 0·4 μmol $NADP^+$ by the undialysed preparation (Fig. 1) could not be related only to the activity of HMPS enzymes and isocitrate dehydrogenase, for the following reasons: (i) the concentrations of glucose, isocitrate and G-6-P were far too low to be responsible for the formation of 400 nmol NADPH; (ii) the substrates of G-6-P regeneration, i.e. G-1-P and glucose (with or without ATP) had no effect on $NADP^+$ reduction (Fig. 4); and (iii) negative feeback inhibition of the NADP reducing enzymes of HMPS did not affect the fast formation of NADPH. We may therefore assume that lymphocytes possess an unknown dialysable $NADP^+$ reducer which seems to play an additional role to HMPS in the reduction of $NADP^+$. Since the level of $NADP^+$ reduction obtained with 0·4 μmol NADP could not be increased by raising the amount of $NADP^+$, it is assumed that 0·4 μmol/5×10^7 cells is the concentration of the $NADP^+$ reducer.

Since lymphocytes have been found by us (Klein, Chan & Malkin, 1986) and others (Kiss & Schuler, 1963; McHaffie & Wang, 1967; Wang, Marquardt & Foker, 1976; Peterkofsky & Prather, 1983) to depend on glycolysis, there is the question whether lactic acid could act as the electron donor for $NADP^+$ reduction (Evans & Karnovsky, 1961; Suzuki & Kakinuma, 1983). This was examined by adding either lactate or pyruvate (as a possible inhibitor) to the assay system and measuring the capacity of the cells to reduce $NADP^+$. Lactate was ineffective, whereas pyruvate prevented the appearance of the NADPH peak (Fig. 5). This last phenomenon was thought to be due to oxidation of the NADPH rather than inhibition of formation by pyruvate, because the NADPH peak (obtained by either the addition of $NADP^+$ to the undialysed preparation or NADPH to the dialysed preparation) disappeared after the addition of pyruvate, and because lactate was formed.

Berger *et al.* (1982) showed that the $NADP^+$/NADPH ratio in resting human lymphocytes was 1·4 whereas in mitogen-stimulated cells it dropped to 0·09. With regard to our present studies we would expect to find most of the nucleotide in its

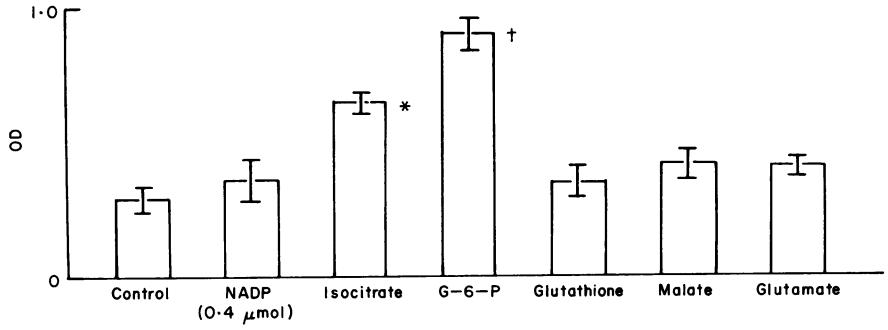

Fig. 4. Reduction of $NADP^+$ by disrupted dialyzed lymphocytes (effect of malate, G-6-P, isocitrate, glutamate, glutathione, glucose and lactate). The preparation was incubated at 20°C for 2 min in 0·7 ml HBSS. Glucose, either with or without 2 μmol ATP (2 μmol, 4 μmol and 11 μmol); G-1-P (1 μmol); and lactate (2 μmol and 6 μmol) added to this preparation, containing 0·4 μmol $NADP^+$, had the same results as the control. $*P < 0.05$; $\dagger P < 0.01$.

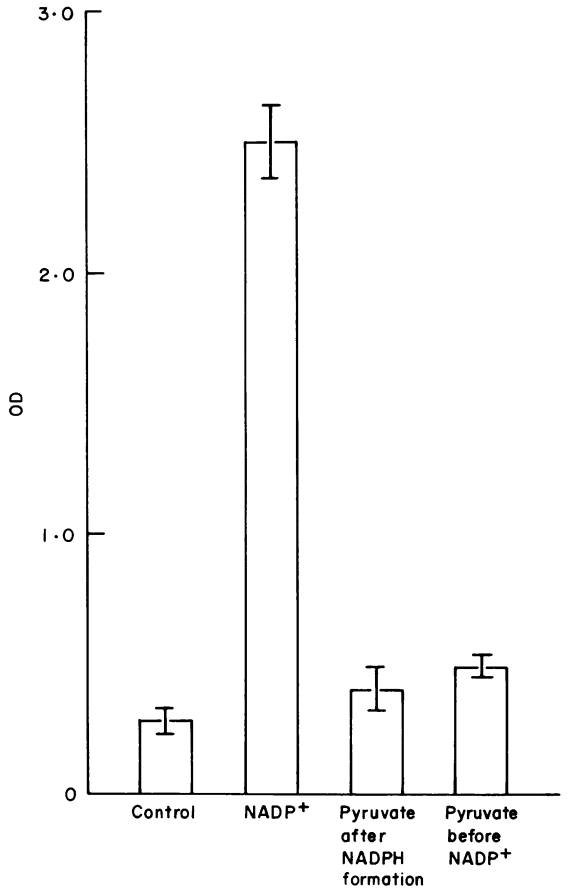

Fig. 5. The effect of pyruvate on $NADP^+$ reduction obtained by undialysed disrupted lymphocytes (cell number and incubation time as described in Fig. 1). $NADP^+$, 0·4 μmol; pyruvate, 2 μmol.

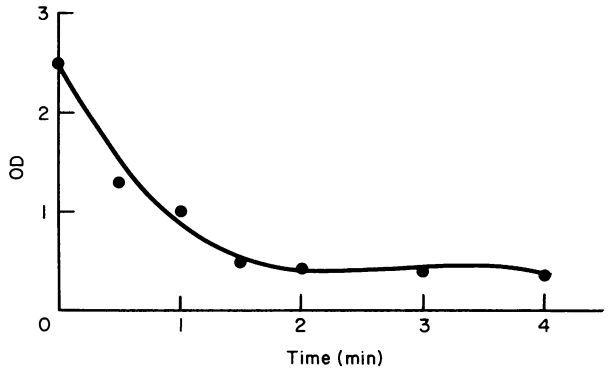

Fig. 6. Time-course of oxidation of formed NADPH (shown in Fig. 5) after the addition of pyruvate (2 μmol).

reduced form. It is possible that the presence of pyruvate in resting cells plays a role in keeping most of the nucleotide in its oxidized form. Pyruvate consumption as a result of an increase in cellular respiration (McHaffie & Wang, 1967; Wang *et al.*, 1976) could perhaps lead to an increase in $NADP^+$ reduction.

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