The presence of NADPH-glyceraldehyde 3-phosphate oxidoreductase in macrophages

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The existence of an NADPH-oxidoreductase which utilizes D-glyceraldehyde 3-phosphate as substrate has been demonstrated in mouse peritoneal macrophages. D-Glyceraldehyde could also serve as substrate, albeit with a 10-fold lower efficiency. No NADH oxidation could be demonstrated with either substrate. Addition of D-glyceraldehyde to cultured macrophages increased the rate of activity of the hexose monophosphate shunt to about 65% of the level observed in zymosan A-stimulated macrophages. The possible involvement of the oxidoreductase in this phenomenon and in the inhibitory effect of D-glyceraldehyde on the production of oxygen free radicals by zymosan-stimulated cells is discussed.

Macrophage *NADPH-triose phosphate oxidoreductase* *Hexose monophosphate shunt*
D-Glyceraldehyde 3-phosphate *D-Glyceraldehyde* *Chemiluminescence*

1. INTRODUCTION

Upon stimulation with soluble or particulate agents, a series of events takes place in macrophages which leads to respiratory burst. This RB is characterized by increased oxygen consumption, stimulation of glucose oxidation via the hexose monophosphate shunt and by a rapid production of oxygen-reactive species [1]. The increased activity of the HMS is attributed to the accelerated rate of regeneration of NADPH to NADP which is an activator of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) [2]. The above reoxidation is carried out mainly by an oxygen reductase which utilizes NADPH while forming oxygen radicals [1]. NADPH may also be odixized in rat skeletal muscle as well as in the rat heart, by an NADPH-dependent oxidoreductase which utilizes D-glyceraldehyde-3-phosphate, a product of the HMS, as substrate [3,4].

The present study was undertaken to determine whether the activity of NADPH-dependent D-GA3P oxidoreductase exists also in macrophages. Such enzyme activity in these cells might gain importance mainly because of the special role of the intracellular NADP/NADPH ratio in stimulated macrophages [5]. The results indicate that NADPH-D-GA3P oxidoreductase is active in macrophages and it may be involved in the activation of the HMS and consequently in the regulation of superoxide generation.

Abbreviations: RB, respiratory burst; HMS, hexose monophosphate shunt; D-GA3P, D-glyceraldehyde-3-phosphate; D-GA, D-glyceraldehyde; Cl, chemiluminescence

2. MATERIALS AND METHODS

2.1. *Materials*

Special materials were obtained from the following sources: D-glyceraldehyde, D-GA3P, dithiothreitol, pyruvate, zymosan A and heparin from Sigma Chemical Co. (St Louis MO);

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[1-^{14}C]glucose, (8.2 mCi/mmol), [U-^{14}C]glucose (310 mCi/mmol) from the Radiochemical Center (Amersham, Bucks); Brewer's thioglycollate broth was obtained from Difco. Other chemicals were of analytical grade.

2.2. *Cell preparation*

Peritoneal macrophages were prepared from C57Bl/6J mice. The cells were collected in Dulbecco's phosphate-buffered saline (PBS) containing 5 IU/ml heparin 4–5 days after an intraperitoneal injection of 2.5 ml sterile thioglycollate. Contaminating red blood cells were lysed by hypotonic treatment and isotonicity was restored by the appropriate volume of 10 × NaCl. Cells were counted and viability was assessed by trypan blue exclusion.

2.3. *Preparation of cultured cells and incubation medium*

Cells were suspended at 2×10^6/ml in PBS containing 5 mM glucose, and 3 ml of this suspension were added to each 50 mm culture dish (Sterilin). After a 90 min incubation at 37°C, the supernatant was decanted, the adherent cells were washed with PBS and incubated in 3 ml PBS supplemented with 5 mM glucose for the periods indicated for each experiment.

2.4. *Preparation of cell extracts*

Macrophages from 3–5 mice were pooled and brought to a final concentration of $5–10 \times 10^7$ cells/ml in 50 mM triethanolamine–HCl buffer (pH 7.4) containing 1 mM dithiothreitol. The extract was prepared by sonicating the cells 3 times at maximal power for 20 s followed by 30 s rest intervals in a Hickville C1125P2 sonicator. The sonicate was centrifuged at $400 \times g$ for 10 min to sediment unbroken cells and nuclei. The resulting supernatant was centrifuged at $105000 \times g$ for 1 h and the supernatant was collected.

2.5. *NADPH-triose phosphate oxidoreductase activity*

This was measured in 50 mM triethanolamine–HCl buffer (pH 7.4) containing 1 mM dithiothreitol, 0.1 mM NADPH and macrophage supernatant (about 1.8 mg protein). No oxidation of NADPH occurred in the absence of triose phosphate. The rate of NADPH oxidation was recorded from the ΔE_{340} and was referred to the amount of proteins in the cell extract.

2.6. *Determination of the levels of activities of the hexose monophosphate shunt and glycolysis*

In order to estimate HMS activity, cultured macrophages were incubated for 30 min (37°C) within [1-^{14}C]glucose (4×10^5 cpm/μmol) and the amount of $^{14}CO_2$ released during the incubation period was determined according to [6]. The rate of glycolysis was determined by adding [U-^{14}C]glucose (1.5×10^5 cpm/μmol) to the incubation medium as in [7,8].

2.7. *Determination of pyruvate*

The total amount of pyruvate produced by the cultured cells was determined as in [9].

2.8. *Luminol-dependent chemiluminescence assay*

The assay was carried out as in [10] with the following minor modifications. The assay mixture consisted of 10^6 cells in 2.5 ml Dulbecco's PBS containing 10 mM Hepes, 5 mM glucose, divalent cations and 10^{-4} M luminol. Chemiluminescence was determined in a Packard tri-carb scintillation counter operating out of coincidence mode.

2.9. *Protein determination*

The amount of proteins in cell extracts or in cultured cells was determined after solubilization (10 min, 100°C in 0.4 M NaOH) as in [11].

3. RESULTS AND DISCUSSION

The initial experiments were aimed at determining whether macrophage extracts possessed an NADPH-dependent triosphosphate oxidoreductase activity. Extracts prepared from macrophages could catalyze the oxidation of NADPH only the presence of D-GA3P (fig.1). The non-phosphorylated compound, D-glyceraldehyde, could also serve as a substrate for the enzyme, albeit with a 10-fold lower efficiency. Addition of 5 mM ATP to the reaction mixture did not affect the rate of NADPH oxidation (not shown). Neither did D-ribose, D-xylose, D-galactose or D-glucose serve as effective oxidizing agents for NADPH. The latter findings exclude the possibility that an aldose reductase is involved in the oxidation of NADPH in peritoneal macrophages [12]. It

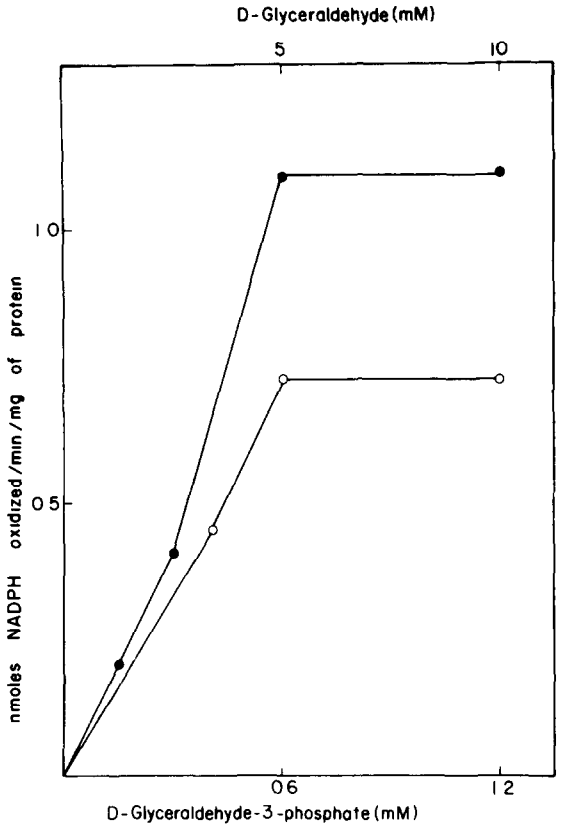

Fig.1. NADPH-dependent D-glyceraldehyde-3-phosphate or D-glyceraldehyde oxidoreductase macrophage supernatant. Details of the procedure are given in section 2. The substrates D-GA3P (○) or D-GA (●) were added to the reaction mixture at the concentrations indicated.

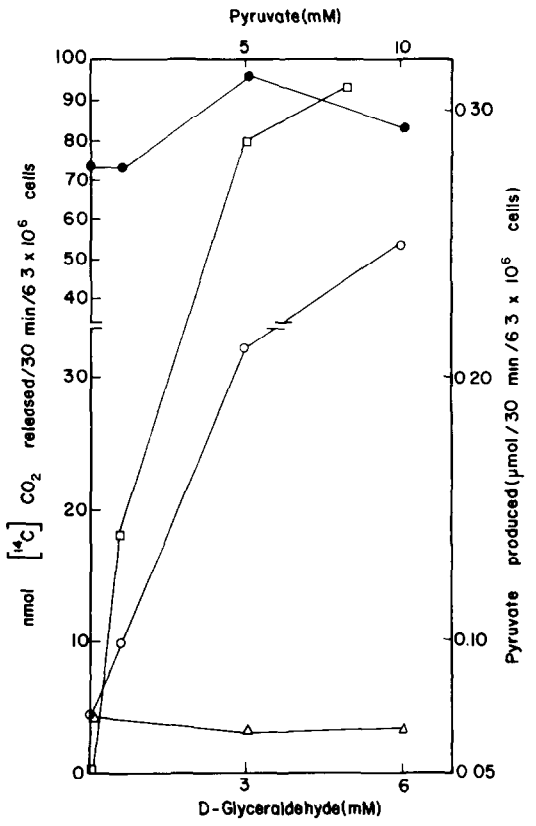

Fig.2. The effect of D-glyceraldehyde and pyruvate on the hexose monophosphate shunt activity and on pyruvate accumulation. The experiments were performed in cultured macrophages. The cells were incubated for 30 min at 37°C. Other details are given in section 2. D-GA (○,●) was added to either resting cells (○) or to cells stimulated by zymosan A, 1 ml 1.0 A_{525}/plate (●). Pyruvate (△) was added to the incubation medium at the concentrations indicated. The amount of pyruvate produced by the cells in the presence of increasing concentrations of D-GA is depicted (□).

should also be pointed out that no oxidation of NADH occurred with D-GA3P or D-GA as substrates (not shown).

HMS activity is one of the main cellular functions affected by the rate of oxidation of NADPH [2]. Thus, it follows that in macrophages NADPH-dependent D-GA3P oxidoreductase activity can possibly serve as an effective means of increasing the rate of HMS. Since D-GA3P does not permeate the plasma membrane, we tested the effect of D-GA on HMS-activity levels in resting and zymosan A-stimulated macrophages. Increasing concentrations of D-GA increased the rate of HMS in non-stimulated cells to about 65% of the level found in cells stimulated with zymosan A (fig.2). Initially, the levels of HMS in stimulated cells were about 18-fold higher than those found in resting cells and D-GA caused only a small further elevation in the levels. These results indicated that the amount of $NADP^+$ available in the stimulated cells was almost saturating for the activity of glucose 6-phosphate dehydrogenase. It is not yet clear whether the intracellular NADPH reduced directly D-GA and/or D-GA3P. However, there are indications that a least part of the D-GA was phosphorylated to D-GA3P in the cells. This suggestion is based on the finding that the addition of

D-GA to cultured macrophages gave rise to large amounts of pyruvate (fig.2). This could occur only if the D-GA was converted to D-GA3P (by an appropriate kinase) which in turn entered the glycolytic flux. Although pyruvate was found to stimulate the HMS in some types of cells [13,14], it had no effect on this pathway in the macrophages (fig.2) and therefore could not account for the stimulatory effect of D-GA on the HMS.

Acceleration of the HMS in resting alveolar macrophages was also observed by depleting the cells of NADPH by the use of paraquat [15]. Non-specific acceleration of the HMS could activate the macrophage in a similar fashion to phagocytosis [16]. Auto-oxidizable dyes such as methylene blue and nitroblue tetrazolium were used for driving the HMS. However, these compounds may also produce reactive species of oxygen by transforming reducing equivalents to oxygen. Thus, at least part of the effect of these compounds may be due to the formation of superoxide. Based on these results, the employment of D-GA as a stimulant of the HMS may serve as an alternative means of probing the question of whether the HMS itself is a signal for macrophage activation.

Stimulation of macrophages is accompanied by a metabolic burst which may be monitored by measuring the generation of oxygen free radicals, particularly superoxide. This radical is generated by a specific NADPH-dependent reductase [17]. We have, therefore, tested the effect of D-GA on the rate of production of reactive oxygen species in the presence and absence of zymosan A by measuring the CL generated by the cells. From the results depicted in fig.3, it can be seen that D-GA did not induce oxygen radical production but actually reduced by 40% the level of oxygen free radicals which was reached by zymosan A stimulation. The latter observation indicates that the rate of regeneration of $NADP^+$ under these conditions is no longer the limiting step in the oxidative branch of the HMS. We suggest that an increased activity of the NADPH-dependent D-GA3P oxidoreductase can potentially compete with the NADPH-dependent oxygen reductase and may thus interfere with the formation of oxygen free radicals.

With regard to the possible physiological significance of the existence of a NADPH-dependent D-GA3P oxidoreductase in the

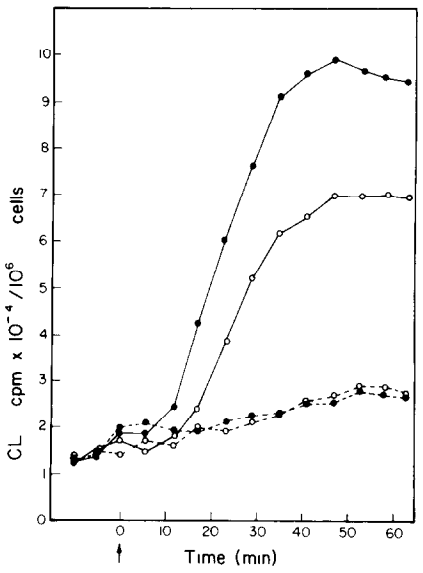

Fig.3. The effects of zymosan A and glyceraldehyde on the response of macrophages as measured by chemiluminescence. Suspended cells (10^6) were preincubated for 20 min in 2.5 ml reaction mixture with or without 5 mM D-GA. At (→) 2 mg zymosan were added to the appropriate cell suspension: (●---●) no D-GA and no zymosan added; (●——●) no D-GA with zymosan; (○---○) D-GA without zymosan; (○——○) D-GA and zymosan added.

macrophages, one could visualize that this enzyme contributes to the overall regulation of the HMS as well as to the control of generation of oxygen radicals via the NADPH-dependent oxygen reductase. It is not yet clear whether the activity of the above enzyme and/or its amount are altered during phagocytosis. However, the rate of production of the substrate, D-GA3P, is probably increased in stimulated cells. D-GA3P can be produced through the non-oxidative branch of the HMS [18] as well as through the glycolytic pathway. As to the rate of glycolysis, it was found to be accelerated by 25% in the presence of zymosan A (not shown). Since the large amounts of ribose-5-phosphate produced via the HMS in the stimulated macrophages, are only partly utilized for the synthesis of purine nucleotides, a significant portion of it is recycled through the non-oxidative branch of the HMS, thus causing an increase in the rate of synthesis of D-GA3P.

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