In vivo operation of the pentose phosphate pathway in frog oocytes is limited by $NADP^+$ availability

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Abstract Evolution of CO_2 from labelled glucose microinjected into frog oocytes in vivo may be ascribed to the pentose-P pathway, as measured by radioactive CO_2 production from [1-^{14}C] and [6-^{14}C]glucose. Coinjection of $NADP^+$ and [^{14}C]glucose significantly stimulated $^{14}CO_2$ production. The effect depends on the amount of $NADP^+$ injected, half maximal stimulation being obtained at 0.13 mM. The increase in CO_2 production was also observed with microinjected glucose-1-P, glucose-6-P or fructose-6-P used as substrates. Phenazine methosulfate, mimicked the effects of $NADP^+$. A high NADPH/$NADP^+$ ratio of 4.3 was found in the cells, the intracellular concentration of $NADP^+$ being 19 μM.

Key words: Glucose metabolism; Pentose-phosphate pathway; $NADP^+$; Frog oocyte

1. Introduction

Frog oocytes can be used as in vivo test tubes for the study of the operation and regulation of glucose metabolism, among other reasons because the controlled variation of intracellular levels of intermediates and cofactors, which cannot otherwise enter the cell, is easily achieved through microinjection.

Glycolysis is operative in grown amphibian oocytes [1], although glucose injected into the cells is mostly utilized for glycogen synthesis [2,3]. Glucose incorporation into the polysaccharide occurs preferentially by the so called indirect pathway, which involves prior degradation of glucose into three carbon compounds which then by gluconeogenesis, regenerate hexoses-P and UDP-glucose, the proximate glycogen precursor [4]. The key gluconeogenic enzymes phosphoenolpyruvate carboxykinase and fructose bisphosphatase are present in oocytes [4,5]. Recently, we have reported an important role of fructose 2,6-bisphosphate in the regulation of glucose metabolism in these cells [6].

A minor portion of the glucose microinjected into frog oocytes (around 5%) is released by the cells as CO_2 mainly through the pentose phosphate pathway [2,7]. This route, which has been considered as an alternative pathway for glucose degradation, diverts some glucose-6-P from glycolysis. We have taken advantage of the properties of the frog grown oocyte [8], which has allowed us to follow the in vivo operation of the pentose phosphate cycle, and to examine under in vivo conditions, the proposition that the level of the oxidized form of $NADP^+$ controls the flux of carbon through the pathway [9–11]. Since in our experimental system the direct microinjection of cofactors and substrates is easily achieved, we have been able to unambiguously demonstrate that in vivo CO_2 production by frog oocytes is accomplished by the pentose phosphate pathway, and that increased levels of $NADP^+$ indeed stimulate the flux of glucose through the oxidative arm of the pentose-P cycle.

2. Materials and methods

2.1. Materials

Glucose, glucose-6-P, glucose-1-P, fructose-6-P, UDP-glucose, phenazine methosulfate, nicotinamide and nicotinamide adenine nucleotides were from Sigma. All labelled compounds were from Amersham. Before the experiment aliquots of radioactive compounds were dried under N_2 to remove ethanol, carrier solutions were added and resuspended in saline to achieve the desired concentration. High performance liquid chromatography using a Dionex CarboPac PA1 column was used to check for partial hydrolysis of metabolite commercial preparations.

2.2. Cells

Stage VI oocytes from freshly killed chilean leptodactylid frogs (*Caudiverbera caudiverbera*) were used. This frog was preferred because of its local availability but mainly because of the large size of its oocytes (2.2 mm diameter). A few experiments with cells from *Xenopus laevis* or *Rana pipiens* showed the same results (data not shown).

2.3. Metabolic labelling

Microinjection procedures, handling of compounds to be injected, and measurement of radioactivity in CO_2 were exactly as described [2]. In short, approximately 50 nl of modified Barth saline [12] containing labelled glucose (or other radioactive precursors), alone or in a mixture with $NADP^+$ or related compounds were injected into oocytes. The incubation of six to eight oocytes was at 22°C under a stream of 100% O_2. Continuous monitoring of CO_2 evolution was performed as described [13]. The nature and specific radioactivity of the labelled compounds are described in the legends to Tables and Figures. For every condition tested, three to five oocytes, microinjected and incubated as zero time controls, were processed through the fractionation protocol. Radioactivity found in these blanks was averaged and subtracted from each experimental value. Results are expressed as carbon radioactivity recovered in CO_2 or as a percentage of control values. We assume that metabolic reactions are initiated by the injection of substrates, i.e. pre-experimental rates and metabolite concentrations are very low in oocytes.

2.4. Determination of reduced and oxidized forms of pyridine nucleotide levels

Pyridine nucleotide levels were measured in non-perturbed oocytes by means of the fluorometric assay described [14]. The excitation wavelength was set at 340 nm and emission at 460 nm was recorded. For the determination of the oxidized forms of nucleotides, groups of 10 oocytes were homogenized in 0.02 N H_2SO_4-0.1 M Na_2SO_4 solution and heated for 30 min at 60°C in order to destroy the reduced nucleotide forms. The homogenate was then neutralized with 1 M Tris-2.5 M NaOH 5:1 and centrifuged at 10 000 rpm for 15 min. An aliquot of the supernatant solution was used to measure NAD^+ plus $NADP^+$ by converting them to their highly fluorescent products (treatment with 6 N NaOH and heating for 20 min at 60°C). Another

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aliquot of the supernatant liquid was incubated with 2.5 mM glucose-6-P and 0.25 units of yeast glucose-6-P dehydrogenase for 30 min at room temperature, in order to convert $NADP^+$ into NADPH. The NAD^+ present was measured in an aliquot of this fraction prior treatment of the sample with 0.2 N HCl followed by heating 15 min at 60°C to destroy NADPH, and subsequent conversion of NAD^+ to the respective high fluorescent product (treatment with 6 N NaOH and heating). Another aliquot of the enzymatic treated sample was used to estimate the NADPH concentration by incubation in 0.05 N NaOH for 15 min at 60°C (which destroys NAD^+); then the remaining NADPH was oxidized and converted to the highly fluorescent product by treatment with 0.03% H_2O_2-6 N NaOH and heating for 20 min at 60°C.

The reduced forms of pyridine nucleotides (NADH and NADPH) were extracted by homogenizing 10 oocytes in 1 ml of 0.045 N NaOH, followed by heating at 60°C for 20 min, neutralization with 1 M Tris-0.1 M HCl 1:1 and centrifugation at 10000 rpm for 15 min. An aliquot of the supernatant solution was treated with 0.03% H_2O_2-6 N NaOH and heated for 20 min at 60°C for measurement of NADH plus NADPH levels. A second aliquot was incubated with 0.4 units of alcohol-dehydrogenase and 20 mM acetaldehyde for 30 min at room temperature in order to oxidize NADH. NAD^+ present in this fraction was determined by conversion to the respective fluorescent probe, the previous destruction of the reduced NADPH, with HCl. NADPH was quantitated in a different aliquot of the enzyme treated fraction to which 0.045 N NaOH was added in order to destroy oxidized NAD^+. After heating, 0.03% H_2O_2-6 N NaOH was added and heated again. Standard curves with NAD^+ and NADH concentrations of 0.05–0.6 μM were used. The standards were prepared at the same time as the homogenates and treated in the same way.

3. Results and discussion

3.1. CO_2 production from labelled glucose

Radioactive CO_2 evolution from labelled glucose was followed in oocytes which were microinjected with the hexose and also in non-perturbed cells to which glucose was added to the external incubation medium. The results obtained show that the efficiency of CO_2 labelling is the same irrespective of whether glucose is microinjected into the oocytes or supplied in the incubation medium. As shown in Fig. 1, most of the label incorporated in CO_2 originates from [1-^{14}C]glucose, while almost no radioactivity is recovered in CO_2 after [6-^{14}C]glucose administration. The pentose-P cycle provides

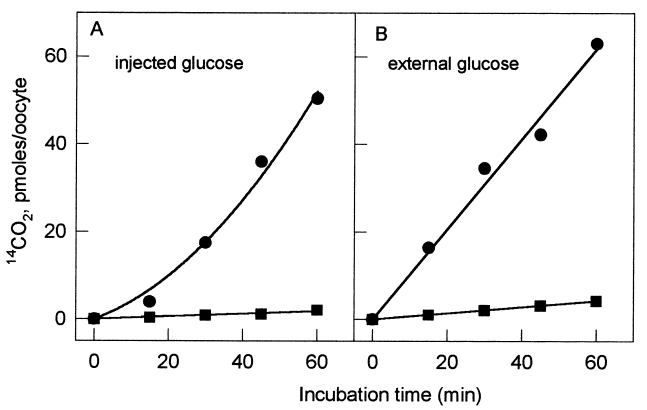

Fig. 1. Time course of CO_2 production from different labelled glucoses. Groups of three oocytes in duplicate flasks were incubated for the indicated times for $^{14}CO_2$ evolution. A: Oocytes were microinjected with 50 nl of saline solution containing 3 nmol (approximately 30000 cpm) of [1-^{14}C]glucose (●) or [6-^{14}C]glucose (■). B: Alternatively, other oocytes were incubated in saline containing 2 mM [1-^{14}C]glucose (●) or [6-^{14}C]glucose (■). The results are expressed as pmol of CO_2 and correspond to the mean of two observations.

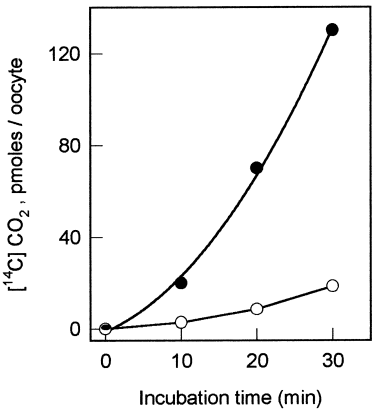

Fig. 2. Time course of CO_2 labelling by frog oocytes microinjected with [1-^{14}C]glucose with or without $NADP^+$.Oocytes were microinjected with 50 nl of saline solution containing 3 nmol of [1-^{14}C]glucose (20000 cpm/nmol) (○). Other oocytes received the same solution plus 3 nmol of $NADP^+$ (●). After injection, groups of three oocytes in duplicate flasks were incubated for the indicated times for $^{14}CO_2$ evolution. Results are shown as the mean of two observations.

the only known mechanism that causes significant differences in the CO_2 outputs from [1-^{14}C] or [6-^{14}C]glucose. Thus, these differences have frequently been used as a measure of the activity of the pathway. However, Larrabee [15] has shown that the C1−C6 difference in CO_2 sets only a lower limit to the flux from glucose to the pentose phosphate pathway, which may in fact be considerably higher. The low ratio C6/C1 obtained in stage VI oocytes suggests that in vivo, CO_2 evolution from glucose may be ascribed almost exclusively to the pentose-P cycle. A similar situation has been reported for *Xenopus laevis* oocytes [7].

3.2. Effect of microinjected $NADP^+$ on CO_2 evolution from [^{14}C]glucose

Expansion of the oocyte pool of $NADP^+$ by direct injection causes a marked stimulation of $^{14}CO_2$ production from labelled glucose. Coinjection of $NADP^+$ and [1-^{14}C]glucose (3 nmol each per oocyte) increased the production of $^{14}CO_2$ about 6–10-fold after 30 min incubation (Fig. 2). The effect was evident as early as 10 min after injection. This result was expected since it is generally accepted that the rate of the pentose-P pathway is not controlled by substrate limitation but by the availability of the oxidized form of $NADP^+$ [11,16,17]. In agreement with these observations, conditions known to augment the $NADP^+$/NADPH ratio result in the stimulation of the rate of the pentose-P pathway, usually measured by the production of $^{14}CO_2$ from [1-^{14}C]glucose [11,18,19]. The very small production of $^{14}CO_2$ from [6-^{14}C]glucose was also stimulated by $NADP^+$ (data not shown).

The increase in $^{14}CO_2$ production depends on the amount of $NADP^+$ injected (Fig. 3). Half maximal effects were obtained with 0.4 nmol $NADP^+$, which assuming a cell volume of 3 μl and uniform distribution of the metabolite inside the cell, is equivalent to 0.13 mM (disregarding endogenous levels).

The influence of glucose concentration on the effects of injected $NADP^+$ was studied by coinjection of different amounts of [U-^{14}C]glucose and a fixed amount (3 nmol per oocyte) of the nucleotide. In every glucose concentration

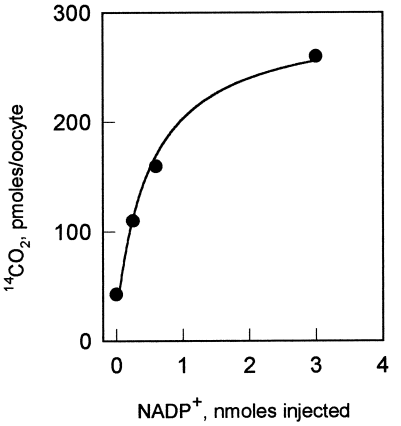

Fig. 3. Effect of the amount of microinjected $NADP^+$ on CO_2 labelling from [1-^{14}C]glucose.Oocytes were microinjected with 48 nl of a saline solution containing 2.9 nmol of [1-^{14}C]glucose (24 000 cpm/nmol) plus the indicated amounts of $NADP^+$. After injection, groups of three oocytes in duplicate flasks were incubated for 20 min. Results are shown as the mean of two observations.

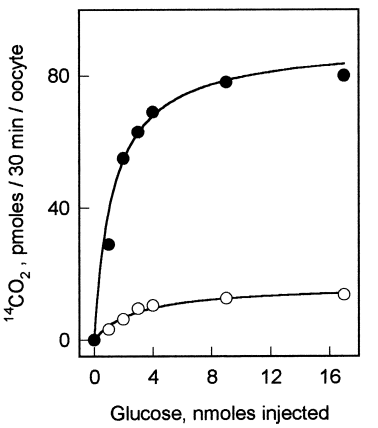

Fig. 4. Influence of the amount of microinjected glucose on the effect of $NADP^+$ on CO_2 labelling. Oocytes were microinjected with 45 nl of saline solutions containing variable amounts (0.9–16.3 nmol) of [U-^{14}C]glucose (○). Specific radioactivity of glucose varied from 4300 to 32 000 cpm/nmol. Other oocytes were injected with the same solutions containing 2.7 nmol of $NADP^+$ (●). Afterwards, groups of three oocytes in duplicate flasks were incubated for 30 min for $^{14}CO_2$ evolution.

tested, $NADP^+$ increased $^{14}CO_2$ formation (Fig. 4). The system reached saturation around 10 nmol (3.3 mM) glucose. The effects of $NADP^+$ were also observed if glucose was substituted by other precursors (Table 1). Thus, $^{14}CO_2$ evolution from labelled fructose-6-P, glucose-6-P or glucose-1-P was stimulated roughly to the same extent by $NADP^+$ (between four and seven times). Production of $^{14}CO_2$ from microinjected glucose-1-P or fructose-6-P may at first seem surprising. However, we have observed that in oocytes microinjected with labelled fructose-6-P, the intermediate is converted within minutes into glucose-6-P (unpublished results). Similarly, we may expect conversion of injected glucose-1-P into glucose-6-P.

3.3. *Specificity of $NADP^+$ stimulatory effect*

Compounds related to $NADP^+$ were injected together with 3 nmol of [U-^{14}C]glucose (Table 2). As already mentioned, $NADP^+$ stimulates $^{14}CO_2$ production about 10-fold. In the presence of NADPH (3 nmol), $^{14}CO_2$ evolution unexpectedly, was slightly increased. A probable explanation for this effect could be an increase in the amount of the oxidized form, as a response of the glutathione system in order to maintain the $NADP^+$/NADPH ratio. The glutathione system is known to exist in amphibian tissues (Dr. A. Venturino, personal communication). NAD^+, NADH or nicotinamide did not stimulate $^{14}CO_2$ production. Phenazine methosulfate, (an artificial electron acceptor dye), mimicked the effects of $NADP^+$ even though only 0.3 nmol per oocyte could be injected (higher amounts precipitated in the injection micropipette). The ability of this and related compounds (pyocyanin, methylene blue) to produce the chemical reoxidation of NADPH, therefore ensuring a continuous supply of $NADP^+$ and increasing the rate of the pathway, has been used in a variety of cells and organs [11,18–23]. The qualitatively similar effect produced by phenazine methosulfate and $NADP^+$ further reinforces the conclusion that the increase of the pentose-P pathway rate is due to the expansion of the pool of the oxidized form of $NADP^+$.

3.4. *Total pyridine nucleotide levels in oocytes*

Intracellular concentrations of oxidized and reduced forms of pyridine nucleotides were measured in extracts from unperturbed oocytes (Table 3). NAD^+ was the most abundant species and the values found were significantly higher than those of $NADP^+$. As in other amphibian species [7], a high

Table 1
The effect of $NADP^+$ on CO_2 labelling from microinjected [U-^{14}C] substrates by frog oocytes

Substrate	$NADP^+$	pmol/oocyte/30 min
[U-^{14}C]glucose	−	13 ± 1.1
	+	52 ± 4.9
[U-^{14}C]glucose-6-P	−	8 ± 1.5
	+	41 ± 4.1
[U-^{14}C]glucose-1-P	−	16 ± 1.3
	+	88 ± 9.1
[U-^{14}C]fructose-6-P	−	16 ± 1.2
	+	112 ± 7.6

Oocytes were microinjected with 45 nl of saline containing 3 nmol (60 000 cpm) of each of the indicated compounds. Other oocytes received in addition 3 nmol of $NADP^+$. Groups of three oocytes in duplicate tubes were then incubated in 50 ml of saline during 30 min. $^{14}CO_2$ evolution was monitored every 10 min. Results are given as the mean ± S.E.M. of six observations.

Table 2
The effect of $NADP^+$ or related compounds on the incorporation of ^{14}C from microinjected [U-^{14}C]glucose into CO_2 by frog oocytes

Compounds coinjected with [U-^{14}C]glucose	CO_2 (% of control values)
None	100 ± 5
$NADP^+$	982 ± 86
NADPH	180 ± 10
NAD^+	109 ± 8
NADH	104 ± 7
Phenazine methosulfate	265 ± 11
Nicotinamide	76 ± 10

Oocytes were injected with 45–50 nl of a saline solution containing 3 nmol of [U-^{14}C]glucose alone or in a mixture with 3 nmol of either of the indicated compounds, except for phenazine methosulfate (only 0.3 nmol). Groups of three oocytes in duplicate tubes were then incubated in 50 ml of saline for 30 min. $^{14}CO_2$ evolution was monitored every 10 min. Results are given as the means ± S.E.M. of the percentage of radioactivity in the controls.

Table 3
Oxidized and reduced pyridine nucleotide levels in frog oocytes

Pyridine nucleotide	pmol/oocyte	Intracelullar concentration (μM)
NAD^+	327 ± 1.5	125.7
NADH	220 ± 10.3	73.5
$NADP^+$	56 ± 12.5	18.8
NADPH	243 ± 9.1	81.2

Groups of 10 non-perturbed oocytes were homogenized and further treated as described in Section 2. Concentrations values were calculated assuming that pyridine nucleotides are uniformly distributed in an intracellular volume of 3 μl. Results are given as the means ± S.E.M. of four observations.

$NADPH/NADP^+$ ratio of 4.3 and a ratio of 1.7 for NAD^+/NADH are maintained by *Caudiverbera* oocytes at this stage of development. As mentioned above, the calculated $NADP^+$ concentration necessary for 50% stimulation of CO_2 production is 130 μM, a value which is around seven times higher than the total intracellular concentration of the nucleotide in the oocyte (19 μM).

It has been reported that NADPH strongly inhibits glucose-6-P dehydrogenase [24–28] and 6-P-gluconate dehydrogenase [24,25,29,30]. The inhibition is competitive with respect to $NADP^+$ and therefore depends on the $NADP^+$/NADPH ratio. These considerations clearly indicate that the activities of the pentose-P pathway dehydrogenases are severely inhibited by the redox conditions generally prevailing in most cells, frog oocytes among them. The activity of glucose-6-P dehydrogenase in oocyte homogenates is 2.1 ± 0.3 U/g wet weight which corresponds to a potential maximal velocity of about 12 600 pmol/min per oocyte [2]. Injected labelled glucose or glucose-6-P at saturating amounts (around 10 nmol/oocyte) produced at most 2–5 pmol/min of labelled CO_2 (not shown). The unperturbed oocyte contains approximately 56 pmol of $NADP^+$ (Table 3). Even though this amount was increased around 50-fold by microinjection, the production of $^{14}CO_2$ was still far below the maximal capacity of glucose-6-P dehydrogenase. We do not know if the oocyte dehydrogenases are inhibited by NADPH, and if all the injected pyridine nucleotide is available for the enzymes. However, the rough calculation suggests that additional factors play a role in maintaining a subdued activity of the pathway.

The stage VI grown oocyte seems to be in a metabolic situation in which, due to the low levels of $NADP^+$ (19 μM) and probable inhibition of the dehydrogenases by NADPH, the pentose phosphate pathway is operating at very low levels in vivo. The pentose phosphate pathway has been shown to operate in vivo in cellular trophoblasts in culture [23]. Our experimental model allows us the study of the pathway in vivo and in a single cell. The controlled variation of the intracellular levels of glucose and $NADP^+$ together with the quantitation of the intracellular levels of $NADP^+$ and NADPH, leads us to the conclusion that in the oocytes, $NADP^+$ availability limits the operation of the pentose phosphate cycle in vivo. The transformation of the oocyte into the mature egg triggers a number of structural and biochemical occurrences. Fluctuations in cAMP levels, changes in pH, protein phosphorylation and dephosphorylation, and ion flux changes have been described [31]. Increased metabolic demands are supposed to be covered by accumulated stores of glycogen and lipids. These changes in energy metabolism have been related to changes in the redox potential of pyridine nucleotides. Thus, variations in the level of such metabolites may play an important role in the regulation of the early events of maturation finally leading to fertilization and cell division.

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