Effect of glucose on ATP dephosphorylation in rat spermatids

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Summary. Round spermatids were isolated from rat testes and the effects of different energy-yielding substrates on the cellular ATP content were estimated. The ATP content was constant and high (6–8 nmol/10⁶ cells) during metabolism of exogenous lactate. During incubation for 30 min in the absence of exogenous lactate, there was a remarkably slow decline of the ATP content, indicating ATP production from other substrates. It was shown that this could reflect β -oxidation of fatty acids, but not the mobilization of an endogenous pool of acetylcarnitine. Glucose metabolism in the absence of exogenous lactate resulted in a rapid decline of the ATP content. This effect of glucose was correlated with a high fructose 1,6-biphosphate content (6–7 nmol/10⁶ cells) and could be prevented by the addition of lactate. It is suggested that metabolism of glucose (and also mannose and fructose, but not galactose) in the absence of exogenous lactate can result in ATP dephosphorylation.

Introduction

Spermatogenesis in rats may be critically dependent on a proper adjustment of the metabolism of energy-yielding substrates, in particular glucose and lactate, by Sertoli cells and spermatogenic cells within the testicular tubules (Jutte *et al.*, 1982; Grootegeod, Oonk, Jansen & van der Molen, 1986). In this connection, the present experiments are concerned with energy metabolism by round spermatids (steps 1–7) isolated from rat testes. Round spermatids (step 1) arise from spermatocytes via meiotic divisions and are transformed eventually into spermatozoa. It has been suggested that rat spermatids may utilize exogenous lactate, rather than glucose, to maintain a high ATP content via mitochondrial oxidation of endogenous pyruvate (Jutte, Grootegeod, Rommerts & van der Molen, 1981; Mita & Hall, 1982; Nakamura, Fujiwara, Yasumasu, Okinaga & Arai, 1982; Nakamura, Okinaga & Arai, 1984; Grootegeod, Jansen & van der Molen, 1984, 1985). In apparent contradiction to this, rat spermatids can convert ³H-labelled glucose to ³H₂O at a considerable rate (Grootegeod *et al.*, 1984) and it has been shown that these cells convert glucose to lactate, although different authors have reported production of greatly different amounts of lactate (Mita & Hall, 1982; Nakamura *et al.*, 1982). The net amount of ATP produced via glucose metabolism by the spermatids is not known.

Bull spermatozoa have been used to study the ATP yield of glycolysis at low and high glucose fluxes (Hammerstedt & Lardy, 1983) and it was observed that a low flux of glucose through the glycolytic pathway is accompanied by substrate cycling and ATP dephosphorylation. Consequently, the ATP yield of glycolysis can be zero or negative, even if the overall ATP yield of glucose metabolism is positive as a result of mitochondrial oxidation of pyruvate originating from glucose. We have tried to determine the effect of glucose metabolism by spermatids on ATP production.

Materials and Methods

Materials. Triosephosphate isomerase, aldolase and glycerol-3-phosphate dehydrogenase (from rabbit muscle), carnitine acetyltransferase (from pigeon muscle), NADH disodium salt grade I and ATP disodium salt special quality were purchased from Boehringer, Mannheim, F.R.G. [$1\text{-}^{14}\text{C}$]Acetyl-CoA (sp. act. 58.7 mCi/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. 2[5(4-Chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) was generously provided by Dr H. S. A. Sherratt, Department of Pharmacological Sciences, University of Newcastle-upon-Tyne, U.K. Percoll was from Pharmacia, Uppsala, Sweden. Bovine serum albumin fraction V and deoxyribonuclease (DN-CL) were purchased from Sigma, St Louis, MO, U.S.A. Concentrates ($\times 50\text{--}100$) of vitamins and essential and non-essential amino acids, according to the formula of Eagle's minimum essential medium, were obtained from Gibco, Grand Island, NY, U.S.A. The luciferin-luciferase mixture (Lumit PM) was from Lumac, Meise, Belgium. All other chemicals (analytical grade) were obtained from commercial sources.

Isolation and incubation of spermatids. Round spermatids were isolated in Dulbecco's phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) supplemented with the energy substrate sodium L-lactate (5 mM) but not with glucose. A suspension of spermatogenic cells was prepared from 6 testes of 32–35-day-old rats (Wistar substrain RI-Amsterdam) using collagenase and trypsin at 32°C , as described previously (Grootegoed, Kruger-Sewnarain, Jutte, Rommerts & van der Molen, 1982). After the trypsin treatment, the isolation procedure was continued at room temperature ($20\text{--}22^\circ\text{C}$). The cells were washed twice in Ca^{2+} -free PBS, supplemented with 5 mM-sodium L-lactate and 0.4% (w/v) bovine serum albumin, and subsequently washed once in the same saline which in addition contained Percoll (20% v/v) and deoxyribonuclease (20 $\mu\text{g/ml}$) (centrifugations for 5 min at 150 g). The cells were resuspended in the Percoll-saline solution ($\sim 12 \times 10^6$ cells/ml) and then the spermatids were isolated by Percoll-gradient centrifugation using a modification of the method described by Meistrich, Longtin, Brock, Grimes & Mace (1981). Percoll gradients (10 ml, with a height of ~ 85 mm) were prepared by centrifugation (for 20 min at 10 000 r.p.m. in a Sorvall SS34 rotor; Sorvall, Newtown, CN, U.S.A.) of Percoll (30% v/v) in Ca^{2+} -free PBS containing 5 mM-sodium L-lactate, bovine serum albumin (0.4%, w/v) and deoxyribonuclease (0.1 mg/ml). The gradients were loaded with 1 ml of the cell suspension and centrifuged for 10 min at 2000 r.p.m. in a Sorvall HB4 rotor. The round spermatids sedimented at about 35 mm. The fraction containing the spermatids was collected and the cells were washed once in Ca^{2+} -free PBS (supplemented with lactate and albumin, see above) and twice in the incubation medium. About 8×10^6 spermatids were recovered per gradient. The cellular composition of the spermatid preparations was estimated as described previously (Grootegoed, Grolle-Hey, Rommerts & van der Molen, 1977) and was as follows: round spermatids (steps 1–7) $92 \pm 2\%$, spermatocytes $4 \pm 1\%$, and other cell types $4 \pm 1\%$ (mean $\pm$ s.d. of 6 different cell preparations). Functional integrity of isolated round spermatids from rats can be maintained during incubation at 32°C for at least 20 h (Grootegoed *et al.*, 1982).

The spermatids were incubated in Eagle's minimum essential medium (MEM) (Eagle, 1959) without glucose, supplemented with 0.4% (w/v) bovine serum albumin fraction V, non-essential amino acids, penicillin (10^5 units/l), streptomycin (100 mg/l) and fungizone (1 mg/l). The cells were washed in this medium after isolation (see above) and the energy substrates were added at $t = 0$ of the incubation. However, when indicated the incubation medium also contained L-lactate during the washings which preceded the incubation. In one series of experiments, the incubation medium was Earle's balanced salt solution (Earle *et al.*, 1943) without glucose, supplemented with albumin and antibiotics (see above). All incubations were carried out at 32°C under an atmosphere of 5% CO_2 in air.

Measurement of cellular ATP content. The spermatids were incubated ($0.4\text{--}0.5 \times 10^6$ cells per incubation) and ATP was estimated by the bioluminescent firefly luciferin-luciferase reaction as

described previously (Grootegoed *et al.*, 1984). Under the present experimental conditions the detection limit of this assay was 0.2 nmol/10⁶ cells.

Enzymic determination of fructose 1,6-biphosphate. Metabolites were extracted by addition of perchloric acid to cells and medium ($0.4\text{--}0.5 \times 10^6$ cells per incubation). The amount of fructose 1,6-biphosphate was determined by fluorometric estimation of NADH oxidation after successive addition of glycerol 3-phosphate dehydrogenase, triosephosphate isomerase and aldolase, as described by Maitra & Estabrook (1964). Under the present experimental conditions the detection limit of this assay was 0.6 nmol/10⁶ cells.

Measurement of total and free carnitine. After incubation, the spermatids (3×10^6 cells per incubation) were extracted twice with 0.5 ml ethanol. The precipitate was removed by centrifugation and the combined supernatants were evaporated at 50°C under a nitrogen stream. The residue was dissolved in 0.1 ml water to estimate free carnitine by the radiochemical method of Cederblad & Lindstedt (1972) as modified by Kerner & Bieber (1983), which involves the formation of ¹⁴C-labelled acetylcarnitine during incubation of carnitine and [1-¹⁴C]acetyl-CoA with carnitine acetyltransferase. Total carnitine was assayed as free carnitine after alkaline hydrolysis of acyl- and acetylcarnitine (Golan, Weissenberg & Lewin, 1984). For that purpose, the initial residue was treated with concentrated NH₄OH at 37°C for 30 min, the samples were evaporated at 50°C under a nitrogen stream and the residue was dissolved in water to measure carnitine as described above.

Results

Glucose-induced ATP dephosphorylation

The ATP content of isolated round spermatids reached a high and constant level within 5–10 min after the start of the incubation in medium supplemented with lactate (Fig. 1). This

Fig. 1. ATP content of spermatids during incubation in the presence of lactate, glucose, mannose or galactose. The ATP content of the spermatids was estimated after incubation for 0–30 min in MEM (without glucose). The medium was supplemented at $t = 0$ with one of the following: no further additions (○), 5 mM-D-galactose (△), 5 mM-sodium L-lactate (●), 5 mM-D-glucose (▲), or 5 mM-D-mannose (▼). The results represent the mean of duplicate incubations (the duplicate values differed by no more than 0.6 nmol/10⁶ cells).

Fig. 2. Comparison of the effects of glucose and fructose on the ATP content of spermatids. The ATP content of the spermatids was estimated after incubation for 30 min in MEM (without glucose up to $t = 0$). The medium was supplemented at $t = 0$ with different concentrations of glucose (●) or fructose (○). The results represent the mean $\pm$ s.d. of triplicate incubations.

constant level was higher than the ATP content at $t = 0$, probably because the cells had been washed in medium without lactate before the incubation. The decline of the ATP content during incubation in the absence of lactate and glucose was remarkably slow (Fig. 1). This could indicate ATP production from other substrates (see below). The addition of glucose or mannose to the incubation medium resulted in a rapid and pronounced decline of the cellular ATP content (Fig. 1). Galactose did not exert such an effect (Fig. 1).

The ATP contents observed using 6 different cell preparations were 7.1 ± 0.8 , 0.8 ± 0.2 and 3.9 ± 0.7 nmol/10⁶ cells, after 30 min of incubation in the presence of lactate, glucose and in the absence of these substrates respectively (mean $\pm$ s.d.; $n = 6$) (values significantly different; $P < 0.005$, t test).

The entry of glucose and mannose into the glycolytic pathway involves phosphorylation of these hexoses at the C-6 position by hexokinase, and the isomerization of mannose 6-phosphate to fructose 6-phosphate. Galactose metabolism involves the formation of galactose 1-phosphate by galactokinase, and the conversion of galactose 1-phosphate to glucose 6-phosphate via a specific set of reactions. The results presented in Fig. 1 would indicate that spermatids do not metabolize galactose to glucose 6-phosphate at an appreciable rate.

A marked effect of glucose on the ATP content of spermatids was observed at a concentration as low as 0.01 mM (Fig. 2). For comparison, the glucose concentration in Eagle's minimum essential medium is 5.6 mM. Mannose was as effective as glucose (not shown), whereas fructose exerted an effect on the cellular ATP content comparable to that of glucose only at 100-fold higher concentrations (Fig. 2). Fructose is converted to fructose 6-phosphate by hexokinase, but the K_m of hexokinase for fructose is several orders of magnitude higher than that for glucose (Katzen & Schimke, 1965).

The glucose-induced ATP dephosphorylation was not completely reversible. This is illustrated in Fig. 3, where it is shown that a relatively low ATP content (~ 3 nmol/10⁶ cells) was obtained during an incubation in the presence of lactate, after the cells had been exposed to glucose in the absence of lactate for 20–40 min.

Accumulation of fructose 1,6-biphosphate

After incubation for 30 min in medium containing glucose, the ATP content of the spermatids

Fig. 3. Effect of lactate on the ATP content of spermatids after incubation in the presence of glucose for 20 or 40 min. The ATP content of the spermatids was estimated after incubation for different time periods in MEM (containing glucose). The medium was supplemented at $t = 20$ or 40 min with 5 mM-sodium L-lactate (control ○; lactate added at $t = 20$ min ▼ or at $t = 40$ min ▲). The results represent the mean $\pm$ s.d. of triplicate incubations.

Fig. 4. Effects of lactate on the ATP and fructose 1,6-biphosphate contents of spermatids during incubation in the presence of glucose. The ATP content (□) and the fructose 1,6-biphosphate content (▨) of the spermatids were estimated after incubation for 30 min in MEM (without glucose up to $t = 0$). The medium was supplemented at $t = 0$ with 5 mM-sodium L-lactate and/or 5 mM-D-glucose. The results represent the mean $\pm$ s.d. of triplicate incubations.

was low but the fructose 1,6-biphosphate content was extremely high (6–7 nmol/10⁶ cells) (Fig. 4). The fructose 1,6-biphosphate content was much lower when the incubation medium contained both glucose and lactate. Under these conditions, the cellular ATP content was high (6–8 nmol/10⁶ cells) (Fig. 4).

ATP production in the absence of glucose and lactate

The ATP content of the spermatids had dropped to a very low level after incubation for 120 min in Eagle's minimum essential medium and Earle's balanced salt solution (both media were glucose-free) (1.2 ± 0.1 and 0.5 ± 0.1 nmol/10⁶ cells respectively). The lactate-maintained ATP contents of spermatids in both media were the same (7.0 ± 0.8 and 7.3 ± 0.3 nmol/10⁶ cells respectively;

Table 1. Total and free carnitine contents of spermatids

Incubation time (min)	D-Glucose conc. (mM)	L-Lactate conc. (mM)	Carnitine (nmol/10 ⁶ cells)		
			Total	Free	Total – free
0	0	0	0.39	0.39	0.00
30	5	0	0.41	0.41	0.00
30	0	5	0.41	0.36	0.05
120	0	5	0.40	0.30	0.10
0*	0	5	0.41	0.31	0.10

Values are for single incubations of 3×10^6 cells.

*L-Lactate was present during the washings before incubation.

Fig. 5. Effect of 2[5(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) on the ATP content of spermatids. The ATP content of the spermatids was estimated after incubation for 0–30 min in MEM (without glucose). POCA (1 mM final concentration) was added at $t = 0$ (control ○; POCA ●). The results represent the mean $\pm$ s.d. of triplicate incubations.

mean $\pm$ s.d. of triplicate incubations). These results indicate that ATP production is not maintained during incubation for 120 min in the absence of exogenous glucose and lactate, and that there is no effect of exogenous amino acids under these incubation conditions. The rates of oxidation of glutamine and leucine by isolated round spermatids are very low (Grootegoed *et al.*, 1986).

Acetylcarnitine might serve as a pool of activated acetyl groups (Van Dop, Hutson & Lardy, 1977). The free carnitine content equalled the total carnitine content at $t = 0$ when rat spermatids had been washed in medium without lactate before incubation (Table 1). However, when lactate had been present during the washings before the incubations, or after incubation in the presence of lactate, a small amount of acetyl- and acylcarnitine (total minus free carnitine) was detected (Table 1). Hence, carnitine and acetylcarnitine could be involved in the oxidation of pyruvate, but there is no endogenous acetylcarnitine pool to support ATP production in the absence of glucose and lactate as depicted in Fig. 1.

The ATP content of spermatids, which were incubated in the absence of glucose and lactate, declined more rapidly after addition of the compound 2[5(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) (Fig. 5) ($P < 0.005$ at $t = 10, 20$ and 30 min, t test). In the presence of lactate, there was no marked effect of POCA; the ATP contents after 30 min of incubation were 6.3 ± 0.3 and 5.7 ± 0.1 nmol/10⁶ cells (control and POCA-treated respectively; mean $\pm$ s.d. of triplicate incubations). The CoA ester of POCA is an inhibitor of the β -oxidation of palmitoyl-CoA through inhibition of carnitine palmitoyltransferase I (Turnbull, Bartlett, Younan & Sherratt, 1984). The results indicate that endogenous fatty acids could serve as an energy-yielding substrate for

spermatids, at least in the absence of glucose and exogenous lactate. The capacity of these endogenous reserves, however, is apparently rather small.

Discussion

In the present experiments, the ATP content of round spermatids isolated from rat testes was strongly decreased through addition of glucose whereas a constant and high ATP content could be obtained during incubation in the presence of exogenous lactate. Exposure of the round spermatids to glucose in the absence of exogenous lactate resulted in a very high fructose 1,6-biphosphate content of these cells. These results indicate that the flux of glucose through the glycolytic pathway in rat spermatids could be restricted by a low activity of one or more enzymes which follow after phosphofructokinase in the glycolytic pathway. The amounts of glycolytic intermediates in cells plus medium after incubation of rat spermatids for 60 min in the presence of 10 mM-glucose have been estimated by Mita & Hall (1982) and Nakamura *et al.* (1982). According to these authors, the rate of glycolysis in spermatids is limited by a low activity of aldolase (Mita & Hall, 1982) or regulated by glyceraldehyde 3-phosphate dehydrogenase (Nakamura *et al.*, 1982).

In the present experiments, we observed that the fructose 1,6-biphosphate content of the spermatids was much lower after incubation in the presence of glucose plus lactate, as compared to the fructose 1,6-biphosphate content obtained with glucose alone. Nakamura *et al.* (1984) have reported that fructose 1,6-biphosphate accumulated in rat spermatids during incubation in the presence of glucose (~ 3 nmol/ 10^6 cells after 60 min). Accumulation of fructose 1,6-biphosphate, however, was also observed by these authors in the presence of glucose plus lactate (2.5 nmol/ 10^6 cells after 60 min). Under these incubation conditions, the ATP contents of the spermatids were low (0.4 and 1.0 nmol/ 10^6 cells, after 60 min in the presence of glucose and glucose plus lactate respectively). Nakamura *et al.* (1982, 1984) isolated the spermatids in phosphate-buffered saline supplemented with glucose but not with lactate. This could explain the discrepancy between the results of Nakamura *et al.* (1984) and the present results.

Substrate cycling between fructose 6-phosphate and fructose 1,6-biphosphate can result in a negative net ATP yield of glycolysis (Katz & Rognstad, 1976; Hammerstedt & Lardy, 1983). This may occur in isolated rat spermatids, when the fructose 1,6-biphosphate content is high during incubation in the presence of glucose but in the absence of exogenous pyruvate and lactate. Under these incubation conditions, the ATP loss in glycolysis may not be compensated for by mitochondrial oxidation of pyruvate and other (endogenous) substrates. From the work of Hammerstedt & Lardy (1983) with bull spermatozoa it appears that the net ATP yield of the glycolytic pathway by itself is zero or negative at low flux, but the spermatozoa can produce much ATP through mitochondrial oxidation of pyruvate originating from glucose. In isolated rat spermatogenic cells, however, the amounts of pyruvate and lactate produced from glucose may be too low to maintain a sufficiently high rate of mitochondrial ATP production. Moreover, endogenous pyruvate (originating from glucose) may be converted to lactate which is then released from the cells, rather than being oxidized in the mitochondria (Mita & Hall, 1982; Nakamura *et al.*, 1982, 1984; Grootegeod *et al.*, 1984).

Inskeep & Hammerstedt (1985) have estimated the ATP production from endogenous metabolism in bull spermatozoa, and these authors have suggested that this ATP-yielding endogenous metabolism could include β -oxidation of fatty acids. Similarly, the present results on the effect of POCA indicate that ATP production by rat spermatids in the absence of glucose and lactate may reflect β -oxidation of fatty acids, rather than the oxidation of amino acids or the mobilization of an endogenous pool of acetylcarnitine. The rate of this endogenous metabolism, however, was too low to balance the effect of glucose on ATP dephosphorylation.

The compound α -chlorohydrin exerts a rapid antifertility effect in male rats and other mammals (Ford & Harrison, 1984, 1985). This action is believed to involve selective inhibition of glyceralde-

hyde 3-phosphate dehydrogenase in spermatozoa. There is a striking similarity between glucose metabolism by isolated rat spermatids (see present results) and by α -chlorohydrin-treated ram and boar spermatozoa (Ford & Harrison, 1984, 1985). This similarity concerns the restricted flux of fructose 1,6-biphosphate through the last part of the glycolytic pathway, and the glucose-induced ATP dephosphorylation concomitant with a high fructose 1,6-biphosphate content.

The present results clearly indicate that, to maintain a high ATP content in round spermatids isolated from rat testes, these cells should not be exposed to glucose in the absence of exogenous lactate. In experiments described previously (Grootegoed *et al.*, 1984) the isolated spermatids were washed before incubation in medium containing glucose but no lactate. After incubation of these cells in the presence of lactate, the ATP content was $\sim 4 \text{ nmol}/10^6$ cells as compared to $6\text{--}8 \text{ nmol}/10^6$ cells in the present experiments. Indeed, the present results indicate that the glucose-induced ATP dephosphorylation is not completely reversible. This may be due to a loss of adenine nucleotides at an extremely low energy charge (Ford & Harrison, 1981).

In rat spermatogenic epithelium, Sertoli cells may convert glucose to lactate at a high rate (Robinson & Fritz, 1981; Jutte *et al.*, 1982; Grootegoed *et al.*, 1986), and the concentration of glucose in the tubular fluid could be very low, as indicated by the observation that ram rete testis fluid contains practically no glucose (Setchell & Waites, 1975). The prevailing conditions in the testicular tubules may therefore protect the spermatogenic cells from glucose-induced ATP dephosphorylation.

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