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THE INHIBITORY EFFECT OF VARIOUS FATTY ACIDS ON AEROBIC GLYCOLYSIS IN EHRLICH ASCITES TUMOUR CELLS

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1. Inhibition of glycolysis in Ehrlich ascites tumour cells by saturated fatty acids, added either in form of potassium salts or incorporated into phosphatidylcholine liposomes, increases with the increasing carbon atom chain length and is independent of the concentration within the range of 0.1 to 1.0 mM. In contrast, the inhibition of glycolysis in the cytosolic fraction from Ehrlich ascites cells depends on the concentration of fatty acids.

2. The content of ATP in Ehrlich ascites cells incubated with fatty acids increases with increasing carbon atom chain length, which leads to a crossing-over in the concentrations of pyruvate and 2-phosphoenolpyruvate. Lowering of the sum of both these metabolites by palmitate and stearate points to the inhibition not only of pyruvate kinase but also of other enzymes of early steps of glycolysis.

3. Fatty acids in intact Ehrlich ascites cells inhibit all three key glycolytic enzymes but added to the cytosolic fraction affect mainly the activity of phosphofructokinase. The inhibition of pyruvate kinase by fatty acids is smaller in the cytosolic fraction from tumour cells than from liver and muscles.

Krebs *et al.* [1] in their study on glucose metabolism in the liver found a stimulating effect of fatty acids on gluconeogenesis. Further investigations [2, 3, 4, 5] contributed to the elucidation of this effect by providing evidence that fatty acids in the liver inhibit key enzymes of the glycolytic pathway. In normal tissues, fatty acids of various carbon chain length and degree of unsaturation directly inhibit key regulatory enzymes of glucose catabolism [3] and indirectly, after oxidation, by an increased level of ATP as a negative feedback effector [4].

It seems interesting to check whether fatty acids, inhibitory in normal cells, can also inhibit glycolysis in neoplastic cells which show a high rate of aerobic glyco-

lysis and a decreased Pasteur effect. Tumours are seemingly devoid of the regulatory mechanisms on account of a lower sensitivity of the allosteric glycolytic enzymes to signal molecules, lower concentration of negative effectors, and a simultaneous increase in the concentration of key enzymes to be inhibited [6, 7, 8].

Studies of Nakahara, published over half a century ago [9, 10, 11], demonstrated the inhibitory effect of oleate on tumour growth, and thus suggested its inhibitory effect on glucose catabolism. In continuation of this observation other authors [12, 13, 14, 15, 16, 40] studied the effect of fatty acids on tumour growth and on their possible application in therapy.

Since it has been shown that the increase in glycolytic activity is correlated with the rate of tumour growth [17] it seems justified to suppose that the observed inhibitory effect of fatty acids on tumour growth should influence the glycolytic activity more significantly than in normal tissues. The aim of this study was to determine whether fatty acids can inhibit glycolysis in Ehrlich ascites tumour and to compare the degree of inhibition of key glycolytic enzymes of this tumour and of normal tissues.

MATERIAL AND METHODS

Ehrlich ascites tumour cells were collected on the 10th day following intraperitoneal implantation, normal livers and skeletal muscles were taken from Swiss albino mice. Ehrlich ascites tumour cell pellets, obtained by centrifugation (1 000 *g* for 5 min) of ascitic fluids and washed twice in physiological saline, were used in further procedures. Part of the cell pellet (approximately 0.3 ml of packed cells per ml of the medium) resuspended in Krebs-Ringer phosphate buffer (pH 7.4) fortified with 30 mM Tris and 10 mM glucose (final concentrations) was immediately used for incubation. Another part of the tumour cell pellet as well as of normal liver and skeletal muscles was homogenized with 20 mM Tris/HCl buffer (pH 7.4) containing 115 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA in a Potter-Elvehjem glass homogenizer and centrifuged at 100 000 *g* at 4°C for 15 min in a Spinco preparative ultracentrifuge. The cytosolic fractions obtained (approximately 2 - 8 mg of protein per ml of the medium) were incubated in 50 mM Tris/HCl buffer (pH 7.4), containing 2.5 mM $KHCO_3$, 2.4 mM K_2HPO_4, 6.7 mM $MgCl_2$, 70 mM KCl, 1 mM ATP, 0.3 mM NAD (Le Page buffer) and 10 mM glucose.

The following compounds, chromatographically pure, were added: octanoic acid (Sigma), palmitic and stearic acids (Reachim), oleic acid (International Enzyme Ltd.), phosphatidylcholine (Merck).

Fatty acids were added to the incubation medium either as potassium salts or in liposomes. After dissolving the fatty acid in a small volume of 0.01 M KOH, the incubation buffer was added and pH was adjusted to 7.4. Lipsomes were obtained according to Olson *et al.* [18] by shaking phosphatidylcholine (1 mM) and the fatty acids with Tris/HCl buffer (pH 7.4), the same as used for tissue homogenization. In these pH conditions fatty acids primarily occur in the ionic form.

In metabolic studies, incubations were performed in a water bath at 37°C. At zero time, and after 30 and 60 min, aliquots of the incubation mixtures were added to 0.6 M perchloric acid. After deproteinization, samples were taken for determinations of lactate [19] and glucose [20]. The remaining cell suspensions were centrifuged after 1 h of incubation. The cell pellets were homogenized in cold 0.6 M perchloric acid. The extracts obtained after centrifugation were adjusted to pH 7.4 with solid $KHCO_3$ and used for ATP [21], pyruvate [22], and 2-phosphoenolpyruvate [23] determinations.

Enzymatic studies were made partly on the cytosolic fraction obtained from Ehrlich ascites tumour cells after 1 h incubation with the fatty acids studied and partly on non-incubated cytosolic fraction of tumour cells, normal livers and skeletal muscles. Hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.2.4) were determined spectrophotometrically at 340 nm as previously described [6] in conditions ensuring pseudo-zero order kinetics, during a 3 min period at 1 min intervals.

Protein was assayed according to Lowry *et al.* [24] The results were analysed statistically using Student's t-test.

RESULTS AND DISCUSSION

It is shown (Table 1) that palmitic and stearic acids added to the medium at 1 mM concentration inhibit both glucose utilization and lactate formation by intact Ehrlich ascites tumour cells. The inhibitory effect is slightly greater after application of palmitic acid in liposomes than in the form of potassium salts, although the differences are not significant. Stearic acid shows a greater inhibitory effect on glycolysis than palmitic acid. Within the concentration range of 0.1 to 1.0 mM, the degree of inhibition by fatty acids depends on the length of the carbon atom chain rather than on the fatty acid concentration in the medium. Spector and coworkers [25, 26, 27, 28] demonstrated that utilization of saturated fatty acids by Ehrlich ascites tumour cells rises with the increase in carbon chain length in the sequence: <decanoic acid<lauric acid<palmitic acid<stearic acid. The present study, which shows the greatest inhibition of Ehrlich ascites tumour glycolysis by stearic acid, agrees with Spector's observation of the greatest rate of this acid penetration into the Ehrlich ascites tumour cells.

In contrast to the intact cells, Ehrlich ascites tumour cytosolic fraction shows a concentration-dependent inhibition of glycolysis by fatty acids. This finding suggests that the membrane of the intact cells limits the penetration of fatty acids into the cell and consequently diminishes their effect on glycolysis. The inhibition of glycolysis by fatty acids in the liver cytosolic fraction, measured by Weber and coworkers [2, 3, 4, 5] by lactate formation, was almost complete at very high octanoic acid concentrations (20 - 25 mM).

Although it is generally accepted that the utilization of fatty acids as an energy source, both in anionic and in undissociated forms at pH below 7.4, is preceded by

their simple diffusion across the cell membrane [25, 27, 28, 29], some authors have suggested that the process depends on the presence of glucose and thus on energy supply [29, 30, 31].

Table 1

The effect of fatty acids on lactate production and glucose utilization in Ehrlich ascites tumour cells and their cytosolic fraction

Cells in Krebs-Ringer phosphate buffer and cytosolic fraction in Le Page buffer, pH 7.4 were incubated in a water bath at 37°C for 60 min with fatty acids (1 mM) added in form of potassium salts or in liposomes in the presence of 10 mM glucose. Mean values ±S. D. from 6 experiments are given.

Fatty acid added	Lactate production		Glucose utilization	
	cells	cytosolic fraction	cells	cytosolic fraction
	μmol/h per mg protein		μmol/h per mg protein	
None (control)	1.65± 0.1	0.97± 0.1	0.96± 0.17	1.56± 0.06
	percentage		percentage	
	100.0 ± 6.1	100.0 ±13.4	100.0 ±17.7	100.0 ± 3.8
Potassium salts:				
Stearate	81.6 ± 0.6	62.9 ±17.5	68.7 ±10.4	78.8 ±19.8
Palmitate	90.3 ±13.3	69.1 ±13.4	94.8 ±22.0	88.5 ±22.4
Oleate	86.6 ±17.2	75.2 ±16.5	96.7 ±15.2	87.8 ±10.2
Octanoate	86.5 ± 9.1	— —	106.0 ±10.5	— —
In liposomes:				
Stearic acid	78.1 ±15.1	— —	73.9 ±20.4	— —
Palmitic acid	85.4 ±10.9	— —	77.1 ±20.8	—
Liposomes alone	102.4 ±12.1	— —	101.0 ±12.5	— —

Fatty acids in the form of soluble salts, inhibiting Ehrlich ascites tumour glycolysis, especially at high concentration, may damage the cell membrane or protein structure simply as detergents by diminishing surface tension. The degree of inhibition of aerobic lactate formation by fatty acid ions is comparable with that caused by fatty acid molecules administered in the form of liposomes.

Compounds used as fatty acid carriers were albumin [32, 27] and Tween 80 as emulgator [33]. Weber *et al.* [5] reported, however, that albumin itself increases the activity of key glycolytic enzymes and our preliminary studies revealed that Tween 80 diminishes glycolytic activity and its effect may enhance that of fatty acids when both are used in combination. The introduction of liposomes as carriers for hydrophobic substances has brought new possibilities to study the effect of fatty acids on intact cells [34]. As shown in the present study, phosphatidylcholine liposomes alone do not change glycolysis in intact Ehrlich ascites tumour cells, although they slightly inhibit glycolytic enzymes examined in cytosolic fraction. The latter effect is probably due to hydrophobic interaction of liposomes with enzyme molecules.

Table 2

The effect of 18 C fatty acids in the form of potassium salts on lactate production in cytosolic fraction of Ehrlich ascites tumour (EAT), liver and muscle

Cytosolic fractions were incubated in Le Page buffer, pH 7.4, at 37°C for 60 min with potassium salts of fatty acids 1 mM in the presence of 10 mM glucose. Mean values ±S. D. from 3 experiments are given.

Tissue	Lactate formation		
	Control (μmol/h per mg protein)	In the presence of stearate (% of control)	oleate (% of control)
Liver	0.028±0.001	60.6	84.6
Muscle	0.100±0.014	88.9	94.4
EAT	0.969±0.038	62.9	75.2

Comparative studies on cytosolic fractions of Ehrlich ascites tumour and normal tissues (Table 2) show that the inhibition of lactate production is high in the cytosol of Ehrlich ascites tumour and liver and much smaller in muscles.

Table 3

The effect of fatty acids on the content of ATP, pyruvate and 2-phosphoenolpyruvate in Ehrlich ascites tumour cells

Cells in Krebs-Ringer phosphate buffer were incubated at 37°C for 60 min with the potassium salts of fatty acids (1 mM) in the presence of 10 mM glucose. Mean values ±S. D. from 6 experiments are given.

Fatty acid added	ATP	Pyruvate	2-Phosphoenolpyruvate
None (control)	nmol per mg protein	nmol per mg protein	nmol per mg protein
	9.1± 1.5	1.77± 0.33	1.20± 0.20
	percentage	percentage	percentage
	100.0±16.5	100.0 ±18.64	100.0 ±16.66
Potassium salts:			
Stearate	185.6± 9.9	34.5 ± 8.48	106.6 ±18.32
Palmitate	153.7±11.0	23.2 ± 5.66	141.6 ±22.49
Oleate	121.9±28.5	46.9 ± 8.47	35.0 ±10.00
Octanoate	114.0±13.1	63.1 ±11.94	151.2 ±26.73

ATP, pyruvate and 2-phosphoenolpyruvate were assayed following the incubation of Ehrlich ascites tumour cells with fatty acids (Table 3) in order to elucidate possible mechanisms of fatty acid action on glycolysis. In Ehrlich ascites tumour cells, in spite of the inhibition of glycolytic energy production under the influence

of fatty acids, an increase in ATP concentration is observed. This points to preferential utilization of fatty acids as an energy source. Since this effect is not seen in the mitochondria-free cytosolic fraction, it may be concluded that the accumulation of ATP results from the oxidation of fatty acids, and that ATP as a negative effector inhibits glycolysis by allosteric regulatory enzymes [35]. ATP-mediated inhibition of glycolysis does not exclude an additional direct inhibitory influence of fatty acid molecules on glycolytic enzymes, especially in mitochondria-free cytosolic fractions. This is in accordance with the data reported by other authors that fatty acid molecules can directly inhibit the key glycolytic enzymes, hexokinase, phosphofructokinase and pyruvate kinase [2, 3, 4, 5, 17].

The crossing-over of pyruvate and 2-phosphoenolpyruvate concentrations in Ehrlich ascites tumour cells under the influence of octanoic acid (Table 3) points to the inhibition of pyruvate kinase only. The sum of both metabolites, which remains unchanged indicates that the early steps of glycolysis are unaffected. ATP in this case does not cooperate with octanoic acid in enzyme inhibition since its level remains almost unchanged.

A similar crossing-over, but accompanied by a decrease in the sum of both pyruvate and 2-phosphoenolpyruvate concentrations, observed under the influence of palmitate and stearate, suggests that apart from inhibition of pyruvate kinase, the early stages of glycolysis are also affected, probably by the rise in ATP level. The inhibitory effect of these fatty acids on cell glycolysis seems therefore to be a combination of a direct influence of fatty acid molecules and of accumulated ATP on at least two steps of glycolysis. After incubation with oleate the greatest decrease observed in the sum of pyruvate and 2-phosphoenolpyruvate, without any crossing-over in their concentrations, suggests that the step of glycolysis sensitive to the inhibition by this unsaturated fatty acid is located before pyruvate kinase.

Table 4

The activites of hexokinase, phosphofructokinase, pyruvate kinase and lactate dehydrogenase in the cytosol of Ehrlich ascites tumour after incubation with potassium salts of fatty acids

Cells in Krebs-Ringer phosphate buffer pH 7.4 were incubated at 37°C for 60 min in the presence of 10 mM glucose and fatty acids (0.05 mM final concentration). Mean values ±S. D. from 3 experiments are given.

Enzyme	Control (mU/mg protein)	In the presence of (% of control)	
		stearate	oleate
Hexokinase	44.7± 8.3	41.5	47.5
Phosphofructokinase	72.8± 21.2	67.3	74.8
Pyruvate kinase	7083.0±1817.0	55.9	52.5
Lactate dehydrogenase	7673.0± 909.0	114.3	107.5

Suggestions arising from metabolic studies were confirmed by enzymatic investigations following the incubation of cells with fatty acids, in which all key enzymes of glycolysis were inhibited (Table 4).

Table 5

The effect of fatty acids in the forms of potassium salts (final concentration 0.05 mM) on the activity of some glycolytic enzymes in cytosolic fractions of Ehrlich ascites tumour (EAT) and normal mouse muscle and liver

Mean values ±S. D. from 3 experiments are given

Enzymes and materials	Control (mU/mg protein)	In the presence of stearate (% of control)	oleate (% of control)
Phosphofructokinase			
EAT	40.4± 17.2	108	88
Muscle	35.9± 20.4	89	64
Liver	25.2± 8.9	36	30
Pyruvate kinase			
EAT	3943 ± 970	84	78
Muscle	4552 ±1833	76	74
Liver	435 ± 150	79	74
Lactate dehydrogenase			
EAT	5643 ±1182	90	90
Muscle	6183 ±1203	99	93
Liver	2163 ± 792	106	108

In studies on the effect of fatty acids on glycolytic enzymes in cytosolic fraction the effect of ATP can be excluded. Inhibition of key enzymes in these conditions is therefore markedly smaller than that observed after cell incubation (Table 5). In studies on the effect of stearate on cytosolic phosphofructokinase activity the greatest inhibition is observed in liver and a smaller one in muscle. Tumour cytosolic phosphofructokinase is insensitive to stearate, therefore the inhibition of this enzyme observed in the cytosol of Ehrlich ascites tumour after incubation is most probably caused by accumulated ATP. Oleate, which weakly inhibits cell glycolysis and only slightly rises the cellular level of ATP, has a marked and perhaps more specific inhibitory effect on tumour cytosolic phosphofructkinase. Our observations on the inhibition of tumour phosphofructokinase by oleate are similar to those of other authors [36, 37]. These observations may explain the decrease in the sum of pyruvate and 2-phosphoenolpyruvate concentrations in tumour cells, and consequently the absence of crossing-over of these metabolites at the stage catalysed by pyruvate kinase.

In contrast to the effect of oleate on pyruvate kinase, inhibition of this enzyme activity by stearate is smaller in the Ehrlich ascites tumour cytosolic fraction than

in normal tissues. This might suggest that tumour cells contain another isoenzyme of pyruvate kinase, less sensitive to stearate. The pyruvate kinase with altered regulatory properties has been already shown in tumour material in studies on its abnormal sensitivity to L-cysteine [38]. Such a difference in pyruvate kinase properties might be regarded as another phenotypical marker of tumour transformation.

The inhibition by fatty acids of key glycolytic enzymes after incubation of intact tumour cells seems to be a result of their cooperation with ATP and is therefore much stronger than in the cytosolic fraction containing no mitochondria. In our studies of the effect of fatty acids on pyruvate kinase it cannot be excluded that the activity observed in the liver cytosolic fraction results from the occurrence of enzyme molecules already modified by some negative effectors. As has been shown in previous studies, erythrocyte pyruvate kinase requires preincubation with ATPase or dialysis to restore its greater activity [39]. Almost ten times lower activity of liver cytosolic pyruvate kinase in comparison with muscle or Ehrlich ascites tumour enzymes does not necessarily reflect a lower enzyme concentration, as might be concluded from the activity measured in conditions of the pseudo-zero order kinetics, but may be due to the presence of this enzyme bound to a negative effector, such as ATP or fatty acids. Comparative investigations of pyruvate kinase sensitivity to exogenous fatty acids after enzyme isolation and partial purification will therefore be undertaken.

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