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Metabolism of ketone bodies, oleate and glucose in lymphocytes of the rat

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1. Isolated incubated lymphocytes utilized acetoacetate, 3-hydroxybutyrate or oleate at about 0.5 μmol/min per g dry wt. These rates were not markedly affected by concanavalin A or by starvation of the donor animal. When ketone bodies replaced glucose in the culture medium, they could not support lymphocyte proliferation when cells were cultured for 48h. 2. Addition of oleate (0.5mM) to isolated lymphocytes increased the rate of O_2 consumption markedly, suggesting that it could contribute about 30% to O_2 consumption. The rate of oleate uptake and the stimulated rate of O_2 consumption were maximal at 0.5M-oleate; this is in contrast with the effect in some other tissues, in which the rate of fatty acid oxidation is linear with concentration up to about 2mM. Since the normal plasma concentration of fatty acid in the fed state is about 0.5mM, this suggests that lymphocytes can utilize fatty acids at a maximal rate in the fed state. 3. Ketone bodies or oleate decreased the rate of glucose utilization by incubated lymphocytes; ketone bodies decreased the rate of pyruvate oxidation and increased the intracellular concentration of hexose monophosphate and citrate, suggesting that 6-phosphofructokinase is inhibited by citrate, and hexokinase by glucose 6-phosphate. These effects may be important not so much in conserving glucose in the whole animal but in maintaining the concentrations of glycolytic intermediates necessary for biosynthetic processes during proliferation.

The importance of glucose metabolism in provision of energy for lymphocytes has been known for some time (for review see Hume & Weidemann, 1980) and the importance of glutamine has been established more recently (Ardawi & Newsholme, 1982, 1983*a*,*b*, 1984). In contrast, the rates of utilization of long-chain fatty acids or ketone bodies and their quantitative importance for energy production in lymphocytes are unclear. For instance, it is not known how far ketone bodies or long-chain fatty acids can support the proliferative response of lymphocytes in the absence of glucose. The work of Lengle *et al.* (1978) suggests that 50–90% of the ATP requirement of cultured lymphocytes could be obtained from the oxidation of oleate. In incubated rat spleen slices, in the presence or absence of glucose, the oxidation of endogenous triacylglycerols contributed significantly to energy production (Suter & Weidemann, 1975). In contrast, the rapidly dividing cells of the intestinal mucosa utilize glutamine and ketone bodies, but not long-chain fatty acids, for energy formation (for review, see Windmueller, 1980).

In the present study, the rates of utilization of acetoacetate, 3-hydroxybutyrate or oleate by isolated rat lymphocytes, together with their effects on rates of glucose metabolism, have been investigated. In addition, the effects of concanavalin A, which stimulates mitogenesis, and starvation of the donor animal on the utilization rates have been studied.

Experimental

Animals

Male Wistar albino rats (160–180g) were obtained from Batin and Kingman, Grimston, Hull, N. Humberside, HU11 4QE, U.K.

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London), London W5 2TZ, U.K., except for the following: D-glucose, glycine, scintillants and all inorganic reagents were ob-

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tained from Fisons Scientific Apparatus, Loughborough, Leics. LE11 0RG, U.K.; concanavalin A, oleate, serum albumin (fraction V) and constituents for the RPMI 1640 medium were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K.; Repelcoté and silicone oil were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K.; cell-culture plates, streptomycin, penicillin and foetal bovine serum were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. The serum was dialysed as described previously (Ardawi & Newsholme, 1983*a*). Radiochemical compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of lymphocytes

Lymphocytes were prepared from rat mesenteric lymph nodes as described by Ardawi & Newsholme (1982).

Incubation procedures

All incubations were performed as described previously, except that higher cell numbers (1×10^8–2×10^8) were used (see Ardawi & Newsholme, 1983*a*). When [1-^{14}C]oleate was used, lymphocytes (5×10^7 cells) were incubated in 10ml silicone-treated Erlenmeyer flasks in a total volume of 1.0ml of phosphate-buffered saline (Culvenor & Weidemann, 1976). Oleate was added as an oleate–albumin complex [the final concentration of albumin was 2.5% (w/v)]. Albumin had been treated with charcoal to remove fatty acids and dialysed before use as described by Chen (1967). The rates of utilization and oxidation of [1-^{14}C]oleate were measured as described by Whitelaw & Williamson (1977), except that $^{14}CO_2$ was trapped on glass-fibre discs (Whatman GF/C, 2.1cm diam.) as insoluble $BaCO_3$. For the separate analysis of cells and medium, lymphocytes were separated from the incubation medium by centrifugation through a layer of silicone oil into $HClO_4$ (see Halestrap & McGivan, 1979).

Assay of metabolites

Concentrations of substrates and metabolites in neutralized extracts of cells plus medium were determined spectrophotometrically (with a Gilford recording spectrophotometer, model 240) by enzymic methods: acetoacetate by the method of Mellanby & Williamson (1974); 3-hydroxybutyrate by the method of Williamson & Mellanby (1974); glucose as described by Bergmeyer *et al.* (1974); lactate by the method of Gawehn & Bergmeyer (1974); glucose 6-phosphate and fructose 6-phosphate by the method of Lang & Michal (1974); and citrate by the method of Dagley (1974).

Cell culture

Lymphocytes were cultured and harvested as described previously (Ardawi & Newsholme, 1983*a*).

Measurement of radioactivity

Radioactivity in non-aqueous samples was measured with a scintillant mixture containing 4.0g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl) benzene in 1litre of toluene, and that in aqueous samples with a Triton-based scintillant mixture containing 750ml of Triton-X-100, 6.0g of 2,5-diphenyloxazole and 0.15g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1.5litres of toluene. Radioactivity was measured in a Beckman liquid-scintillation counter (model L7500).

Expression of results

Changes in concentrations of substrates or metabolites during the incubation were determined from the net change between zero time and 60min incubation. Rates of substrate utilization or metabolite production are expressed (unless otherwise indicated) as μmol/h per g dry wt. of cells, calculated on the basis that 10^{10} cells are equivalent to 0.815 ± 0.07g dry wt. after drying at 50°C for 16h.

Results and discussion

Ketone-body metabolism and the effect of concanavalin A

Lymphocytes utilized acetoacetate at a rate of about 0.53μmol/min per g dry wt.; this rate was linear with time up to 40min. Some acetoacetate was converted into 3-hydroxybutyrate (Table 1). This rate is considerably lower than that reported for rat thymocytes (Hume *et al.*, 1978), enterocytes (Hanson & Parsons, 1978) or colonocytes (Roediger, 1982). Lymphocytes utilized 3-hydroxybutyrate at a similar rate to that of acetoacetate, and some acetoacetate was produced (Table 1). The net amount of acetoacetate utilized was sufficient to account for about 70% of the O_2 consumed by the incubated lymphocytes (see Ardawi & Newsholme, 1983*a*). However, this value may be too high, since some of the acetoacetate that is utilized could be converted into fatty acids; lipogenesis was not measured in the present work.

Starvation of the donor rats for 48h caused no significant change in the rate of ketone-body utilization by isolated lymphocytes (results not shown). Concanavalin A did not stimulate markedly the rate of acetoacetate utilization (Table 1), unlike the effect on glucose or glutamine utilization (Ardawi & Newsholme, 1983*a*), nor did it affect the

Table 1. *Effects of concanavalin A and glucose on the metabolism of ketone bodies by incubated mesenteric lymphocytes of the rat*

Lymphocytes were incubated for 60 min in 1 ml of incubation medium in the presence or absence of concanavalin A (30 μg/10^7 cells) and substrate(s) as indicated. Rates of utilization (indicated by minus sign) or production are presented as means ± S.E.M., with the numbers of experiments with separate cell preparations given in parentheses. Statistical significance of difference between control and experimental (Student's *t* test, based on difference between means) is indicated by *($P<0.05$) or **($P<0.001$).

Substrate added	Concanavalin A	Rates of utilization or production (μmol/h per g dry wt.) Acetoacetate	3-Hydroxybutyrate	Glucose	Lactate
Acetoacetate (3mM)	−	−31.83 ± 2.72 (10)	8.00 ± 0.44 (6)	−	10.70 ± 1.31 (7)
	+	−38.90 ± 2.00 (8)	7.74 ± 0.60 (6)	−	−
3-Hydroxybutyrate (3mM)	−	7.19 ± 0.80 (5)	−27.44 ± 2.85 (12)	−	17.26 ± 3.10 (6)
Glucose (5mM)	−	−	−	−35.21 ± 1.85 (7)	52.50 ± 2.73 (7)
	+	−	−	−54.28 ± 3.11 (9)**	101.52 ± 2.90 (9)**
Acetoacetate (3mM) plus glucose (5mM)	−	−32.80 ± 1.70 (10)	8.91 ± 0.50 (6)	−27.56 ± 5.30 (7)*	60.82 ± 3.40 (7)
	+	−39.00 ± 2.12 (6)	7.86 ± 0.70 (5)	−48.37 ± 1.50 (7)**	109.44 ± 8.40 (5)**

conversion of [^{14}C]acetoacetate into $^{14}CO_2$ (results not shown). The marked stimulation of the rates of glucose utilization and lactate production caused by concanavalin A were still observed in the presence of acetoacetate (Table 1). In addition, although the rate of ketone-body utilization is high, it cannot support lymphocyte proliferation when cells were cultured for 48 h (that is when glucose was replaced by either 3-hydroxybutyrate or acetoacetate in the culture medium; Table 2).

Oleate metabolism and the effect of concanavalin A

The rate of [1-^{14}C]oleate utilization by incubated lymphocytes was about 0.56 μmol/min per g dry wt. (Table 3), which was linear with time between 20 and 60 min (results not shown). (Isolated rat hepatocytes utilized oleate at a rate about 4-fold greater than this; Whitelaw & Williamson, 1977.) This rate was unchanged when lymphocytes were isolated from 48 h-starved rats. The rate of conversion of [1-^{14}C]oleate into $^{14}CO_2$ accounts for about 1% of the total oleate utilized by the cells (Table 4). However, addition of oleate (0.5 mM) to isolated lymphocytes increases O_2 consumption by about 40% above that with endogenous substrate(s); this suggests that the fatty acid can contribute about 32% to O_2 consumption of the isolated lymphocytes, in comparison with that of glucose and glutamine (see Ardawi & Newsholme, 1983*a*). The apparent low rate of oxidation as indicated by conversion of [1-^{14}C]oleate into $^{14}CO_2$ may be due to marked dilution of the radiolabelled fatty acid intracellularly. A high rate of turnover of phospholipids could account for this dilution. About 50% of the oleate that was utilized appeared in esterified form in the lymphocytes (Table 4). (No further characterization of various esters was performed.)

Neither the rate of oleate utilization (Fig. 1) nor

Table 2. *Effects of ketone bodies on [^{3}H]thymidine incorporation in concanavalin-A-stimulated rat lymphocytes in cultures*

Cells (5×10^5) were cultured in RPMI 1640 medium (free of glucose) as described in the Experimental section. Glucose, acetoacetate or 3-hydroxybutyrate were added at the start of the cell culture. The values shown are means ± S.E.M., with the numbers of separate experiments given in parentheses.

Addition to culture medium	[^{3}H]Thymidine incorporation (c.p.m./5×10^5 cells)
None	2198 ± 68 (8)
Glucose (2mM)	73418 ± 816 (10)
Acetoacetate (2mM)	2362 ± 326 (10)
3-Hydroxybutyrate (2mM)	2511 ± 380 (10)

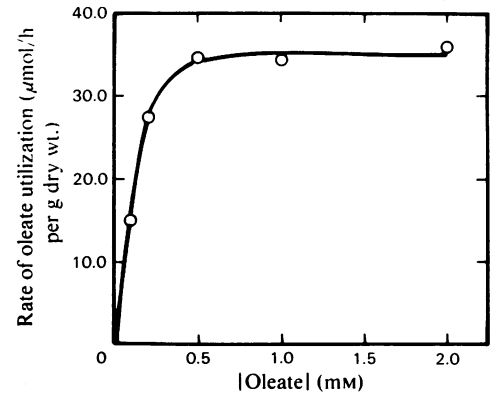

Fig. 1. *Oleate utilization by incubated lymphocytes at different oleate concentrations*

Lymphocytes were incubated as described in the Experimental section in the presence of various oleate concentrations as indicated. Results are means of at least three separate experiments.

Table 3. *Effects of concanavalin A and glucose on the metabolism of [1-^{14}C]oleate by incubated mesenteric lymphocytes of the rat*

Details of incubation with [1-^{14}C]oleate are given in the Experimental section. Concanavalin A was added at 150 μg/ml as indicated. Rates of utilization (indicated by minus sign) or production are presented as means ± S.E.M., with the numbers of experiments with separate cell preparations given in parentheses. Statistical significance of difference between control and experimental values (Student's *t* test, based on difference between means) is indicated by *($P<0.05$) or **($P<0.001$).

Substrate added	Concanavalin A	Rates of utilization or production (μmol/h per g dry wt.) Oleate	Glucose	Lactate
[1-^{14}C]Oleate (0.5 mM)	−	−34.91 ± 0.90 (6)	−	12.61 ± 2.50 (4)
	+	−36.86 ± 1.45 (6)	−	17.23 ± 3.10 (4)
Glucose (5 mM)	−	−	−34.85 ± 1.31 (4)	52.80 ± 1.40 (4)
	+	−	−51.63 ± 3.72 (5)**	98.70 ± 2.50 (5)**
[1-^{14}C]Oleate (0.5 mM) plus glucose (5 mM)	−	−33.84 ± 1.0 (6)	−30.37 ± 0.70 (5)	66.06 ± 1.61 (5)
	+	−37.20 ± 2.7 (5)	−49.61 ± 2.65 (5)**	107.80 ± 5.20 (5)**
[1-^{14}C]Oleate (1.0 mM) plus glucose (5 mM)	−	−34.10 ± 2.2 (4)	−26.55 ± 1.20 (5)*	48.32 ± 5.50 (5)

Table 4. *Percentage distribution of [1-^{14}C]oleate utilized by incubated lymphocytes*

For experimental details, see the Experimental section. The distribution of ^{14}C radioactivity is presented as percentage (± S.E.M.) of the total radioactivity recovered in all fractions, for six separate experiments.

Addition to incubation	Distribution of [1-^{14}C]oleate (%) Esterified fats	Metabolic CO_2	Unaccounted for
[1-^{14}C]Oleate (0.5 mM)	50.00 ± 4.70	0.70 ± 0.07	49.30 ± 6.30
[1-^{14}C]Oleate (0.5 mM) plus concanavalin A (150 μg/ml)	62.63 ± 2.71	0.85 ± 0.05	36.52 ± 4.53

the rate of oxygen uptake (results not shown) was affected by increasing oleate concentration from 0.5 to 1.5 mM; this could be due to the use of only 2.5% (w/v) albumin in the incubation medium. However, it is in complete contrast with the situation known to exist for other tissues [e.g. liver (Fritz *et al.* , 1958), heart (Opie, 1968), kidney (Weidemann & Krebs, 1969) and skeletal muscle (Hagenfeldt, 1979)], in which the rate of fatty acid oxidation markedly increases as the concentration increases above 0.5 mM. Furthermore, the rate of uptake of non-esterified fatty acid by the heart exhibits a threshold effect: below about 0.35 mM very little or no fatty acid uptake occurs (see Opie, 1968), and this may also be the case for skeletal muscle (see Hagenfeldt, 1979). The plasma fatty acid concentration in the normal fed animal (rat or man) is about 0.5 mM and increases towards 2 mM in prolonged starvation (Eaton *et al.*, 1969). Hence fatty acids may be utilized and oxidized by lymphocytes even in the fed state. The significance of this may be to enable fatty acids to be removed from the bloodstream even in the fed animal to support lymphocyte proliferation and growth. Fisher & Mueller (1971) have shown that the net synthesis of membrane lipids in lymphocytes begins about 6 h after stimulation, when the plasma fatty acid concentration may only be 0.5 mM.

Stimulation of lymphocytes *in vitro* by concanavalin A did not change the rate of oleate utilization (Table 3), but the rate of oxidation was increased and the percentage of [1-^{14}C]oleate recovered as esterified fats increased by about 25% (Table 4).

Effects of acetoacetate or oleate on glucose metabolism

Acetoacetate or oleate (1 mM) decreased the rate of glucose utilization (Tables 1 and 3); acetoacetate also increased the rate of lactate formation (Table 1). In addition, preliminary experiments using [5-^{3}H]glucose have indicated a decrease in the rate of glycolysis as measured by $^{3}H_2O$ formation in the presence of ketone bodies (results not shown). These effects are consistent with the predictions of the 'glucose–fatty acid–ketone body cycle' (for review, see Newsholme & Leech, 1983)

The mechanism by which acetoacetate may regulate glucose utilization was investigated by studying the changes in the concentrations of glycolytic intermediates. The presence of acetoacetate increased the concentration of both glucose 6-phosphate and fructose 6-phosphate, suggesting that the activity of 6-phosphofructokinase was

Table 5. *Effect of acetoacetate on the concentration of hexose monophosphates and citrate in incubated mesenteric lymphocytes of the rat*

For details of incubation procedure see the Experimental section. Where indicated, 5mM-glucose or 5mM-glucose plus 3mM-acetoacetate were present in the incubation medium. Concentrations are presented as means ± S.E.M., with the numbers of experiments with separate cell preparations given in parentheses. A statistical significance of difference between glucose and glucose plus acetoacetate incubations (paired *t*-test) is indicated by *($P<0.05$).

	Concn. (μmol/g dry wt.)		
Addition to medium	Glucose 6-phosphate	Fructose 6-phosphate	Citrate
Glucose (5mM)	0.193 ± 0.03 (8)	0.054 ± 0.01 (6)	4.98 ± 1.14 (6)
Glucose (5mM) plus acetoacetate (3mM)	0.262 ± 0.02 (8)*	0.072 ± 0.01 (6)*	6.48 ± 0.57 (7)*

Table 6. *Effects of ketone bodies on the rate of oxidation of [1-^{14}C]pyruvate by mesenteric lymphocytes of the rat*

For details of incubation procedure see the Experimental section; 2mM-[1-^{14}C]pyruvate alone or plus acetoacetate and 3-hydroxybutyrate or both were added as indicated. Produced $^{14}CO_2$ was collected as described in the Experimental section. Results are presented as means ± S.E.M., with the numbers of experiments with separate cell preparations given in parentheses. Statistical significance of difference between incubations of pyruvate and pyruvate plus acetoacetate or 3-hydroxybutyrate or both (Student's *t* test, based on difference between means) is indicated by *($P<0.001$).

Addition	Rates of [1-^{14}C]pyruvate oxidation (μmol/min per g dry wt.)
None	0.60 ± 0.05 (8)
Acetoacetate (3mM)	0.38 ± 0.03 (6)*
3-Hydroxybutyrate (3mM)	0.40 ± 0.02 (6)*
Acetoacetate (3mM) plus 3-hydroxybutyrate (3mM)	0.33 ± 0.03 (5)*

decreased. This could have been caused by the increased concentration of citrate (Table 5). The increased concentration of glucose 6-phosphate would be expected to inhibit hexokinase activity, and hence lead to a decrease in the rate of glucose utilization.

Inhibition of the activity of pyruvate dehydrogenase by the presence of acetoacetate was indicated from the marked decrease in the rate of conversion of [1-^{14}C]pyruvate into $^{14}CO_2$ in the presence of acetoacetate (Table 6). This may have been caused by an increase in the [acetyl-CoA]/[CoA] ratio in the mitochondria.

It is noteworthy that 0.5mM-oleate increased the rate of lactate production, but there was no such effect when 1.0mM-oleate was used (Table 3). This may be explained by the lower concentration of oleate inhibiting pyruvate oxidation only, whereas the higher concentration may inhibit the rates of both pyruvate oxidation and glycolysis (at the 6-phosphofructokinase reaction).

In view of the fact that ketone bodies decrease glucose utilization and pyruvate oxidation, it is somewhat surprising that starvation of the donor animal increases the rate of glucose utilization in incubated lymphocytes (Ardawi & Newsholme, 1983*a*). However, it is unlikely that the decrease in glucose utilization caused by ketone bodies in tissues of the immune system (spleen, thymus and lymph nodes) will be physiologically important; the rate of utilization will be very small in comparison with that of other tissues (heart, brain, skeletal muscle). Consequently the effect of ketone bodies may be not so much to decrease glycolytic flux in order to conserve glucose for other tissues but to maintain normal or even elevated concentrations of glycolytic intermediates for biosynthetic purposes.

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