Interactions of insulin and dexamethasone in the control of pyruvate kinase activity and glucose metabolism in sheep adipose tissue

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1. The $V_{\max}$ activity of pyruvate kinase of sheep adipose tissue increased during tissue culture up to 48 h; the increase was blocked by actinomycin D (an inhibitor of transcription) and was promoted by insulin and antagonized by dexamethasone. 2. In contrast with their effects on pyruvate kinase, insulin and dexamethasone acted synergistically to increase the activity of glucose-6-phosphate dehydrogenase of sheep adipose tissue maintained in culture. 3. Insulin stimulated, whereas dexamethasone inhibited, glucose utilization by sheep adipose tissue maintained in culture; the two agents were mutually antagonistic, and their effects were prevented by actinomycin D. 4. Antimycin A (an inhibitor of the electron-transport chain) stimulated glucose uptake and lactate output by sheep adipose tissue in the presence of dexamethasone and insulin, suggesting that the effects of dexamethasone on glucose utilization by sheep adipose tissue were not due to an inhibition of glucose transport. 5. Comparison of these findings with previous studies on the endocrine control of hepatic pyruvate kinase shows that there are major differences in the control of these $V_{\max}$ activities in liver and adipose tissue. 6. Although glucocorticoid hormones inhibit glucose utilization themselves and can antagonize the stimulatory effects of insulin on glucose utilization in adipose tissue from both sheep and rats, there appear to be major differences in the sites of action of these hormones in the two species.

INTRODUCTION

Almost all dietary carbohydrate is converted into short-chain fatty acids (e.g. acetate) in the rumen of sheep and other ruminants, so most of their glucose requirement has to be met by gluconeogenesis (see Ballard *et al.*, 1969). Consequently the control of glucose utilization is more critical in ruminants than in species such as the rat. Thus adipose tissue of sheep and cattle differs markedly from that of the rat, in that acetate rather than glucose is the major precursor of fatty acid synthesis and in the poor response of its metabolism to insulin during short-term (2–3 h) incubations *in vitro* (see Vernon, 1980).

Adipose tissue from ruminants does use some glucose, the major products of its metabolism being lactate, acylglycerol glycerol and CO_2 (Khachadurian *et al.*, 1966; Yang & Baldwin, 1973; Robertson *et al.*, 1982). When adipose tissue from sheep was maintained in tissue culture for 24 h, the rate of glucose utilization increased; this was accompanied by an increase in pyruvate kinase activity, whereas the activity of a number of other enzymes, including hexokinase and phosphofructokinase, did not change (Robertson *et al.*, 1982). A selective increase in pyruvate kinase activity has also been observed when human adipose tissue is maintained in tissue culture (Smith *et al.*, 1976). In contrast with its lack of effects during short-term incubations, insulin stimulated glucose uptake and also increased pyruvate kinase activity of bovine adipose tissue during incubations for 24 h or longer (Vernon *et al.*, 1985).

Pyruvate kinase occurs as several isoenzymes. The isoenzyme of sheep adipose tissue has not been fully characterized, but its kinetics with respect to phospho-

enolpyruvate and fructose 1,6-bisphosphate (J. P. Robertson & R. G. Vernon, unpublished work) resemble those of rat adipose tissue (Denton *et al.*, 1979). Pyruvate kinase of rat adipose tissue is the M_2 isoenzyme, which differs from the L isoenzyme of liver in several respects (see Denton & Halestrap, 1979; Claus & Pilkis, 1981). For example, the L isoenzyme of liver is subject to phosphorylation by cyclic AMP-dependent kinase, with a change in properties (see Engström, 1980; Claus & Pilkis, 1981), whereas, although insulin decreases the K_m for phosphoenolpyruvate of the M_2 isoenzyme of rat adipose tissue, this does not involve any change in phosphorylation status (Denton *et al.*, 1979). Again, as described above, the activity of the adipose-tissue isoenzyme increases during tissue culture, whereas that of the L isoenzyme does not increase in hepatocyte culture, even in the presence of insulin (see Cimbala *et al.*, 1985).

The factors (other than insulin) and mechanisms which determine the maximum pyruvate kinase activity of adipose tissue have not been investigated in the sheep or any other species. In the present study we show that both the increase in pyruvate kinase activity and glucose utilization induced by insulin during tissue culture of sheep adipose tissue are blocked by an inhibitor of transcription and are modulated by glucocorticoids.

EXPERIMENTAL

Animals

Sheep were all Finn $\times$ Dorset Horn cross-breeds; they were fed on a cereal mixture (200 g at 8:00 h and 16:00 h each/day) plus hay *ad libitum* for at least

Table 1. Effects of cortisol and theophylline on the stimulation of pyruvate kinase activity by insulin in sheep adipose tissue maintained in tissue culture

Adipose tissue from 7–9-month-old castrated male sheep was maintained in culture in the absence and the presence of the various agents for 24 or 48 h, after which the pyruvate kinase activity ($V_{\max}$) was measured with 3 mM-phosphoenolpyruvate plus 3 mM-fructose 1,6-bisphosphate, and the activity ratio (ratio of activities measured with 0.05 mM-phosphoenolpyruvate and with 3 mM-phosphoenolpyruvate plus 3 mM-fructose 1,6-bisphosphate) was determined. Results are expressed per mg of protein (protein concentration was measured in tissue pieces before and after tissue culture), as means $\pm$ S.E.M., with the numbers of observations in parentheses, and were analysed by Student's *t* test for paired observations; * denotes value significantly different from that of tissue slices maintained in culture in the absence of hormones ($P < 0.05$). Pyruvate kinase activity ($V_{\max}$) before culture was 202 ± 21 (5) nmol/min per mg of protein, and the activity ratio was 0.22 ± 0.02 (5). The 15000 g-supernatant protein concentration of tissue before culture was 1.1 ± 0.2 mg/g of tissue (mean $\pm$ S.E.M. for five values).

Agent added	Time in culture ...	Pyruvate kinase			
		$V_{\max}$		Activity ratio	
		24 h	48 h	24 h	48 h
None		301 ± 84 (5)	945 ± 71 (4)	0.16 ± 0.01 (5)	0.12 ± 0.01 (4)
Insulin (17 nM)		478 ± 68 (5)	1807 ± 200 (4)*	0.15 ± 0.02 (5)	0.12 ± 0.01 (4)
Insulin (17 nM) + cortisol (2.8 μ M)		324 ± 3 (4)	846 ± 249 (3)	0.16 ± 0.02 (4)	0.15 ± 0.03 (3)
Insulin (17 nM) + theophylline (2 mM)		389 ± 46 (5)	1148 ± 90 (4)	0.18 ± 0.01 (5)	0.15 ± 0.01 (4)

1 month before slaughter. A catheter was placed in the jugular vein, and on the next day the sheep were anaesthetized at 10:00 h (Vernon *et al.*, 1981), after which they were exsanguinated, and samples of subcutaneous adipose tissue from the fat-pads immediately anterior to the hind-limbs were removed and placed in 0.15 M-NaCl at 37 °C.

Tissue culture

Pieces of adipose tissue weighing about 5 mg were prepared and maintained in tissue culture at 37 °C in Medium 199 containing Earle's salts, L-glutamine, 25 mM-Hepes, pH 7.3 (Gibco-Biocult, Paisley, Scotland, U.K.), 2 mM-acetate and antibiotics with a gas phase of air/CO₂ (19:1) as described previously (Robertson *et al.*, 1982). Various hormones and agents were also added to some cultures as described in the text and Tables. Bovine insulin, pig glucagon, dexamethasone and antimycin A were from Sigma Chemical Co.; sheep growth hormone, prolactin and thyrotropin were gifts from NIADDK, Bethesda, MD, U.S.A. Dexamethasone (1 mM) was dissolved in ethanol/water (1:1, v/v) and diluted with Medium 199 to the required concentration; the ethanol concentration in the tissue culture medium was 10 μ M. Preparatory experiments revealed that this concentration of ethanol had no effect on the variables measured. However, in experiments in which the effects of dexamethasone were investigated, all control cultures were supplemented with 10 μ M-ethanol.

After 24 or 48 h of culture, the adipose-tissue explants were blotted dry, weighed and frozen in liquid N₂ before determination of enzyme activities. Samples of culture media were taken and stored at –20 °C for assay of glucose, lactate, glycerol and insulin.

Assays

Pyruvate kinase and glucose-6-phosphate dehydrogenase were assayed as described by Robertson *et al.* (1982). Insulin was assayed as described by Vernon *et al.*

(1981). Glucose, lactate and glycerol were determined by using a Titretek Multiskan Analyser. DNA concentration of tissue pieces before culture and protein concentration of the supernatants used for pyruvate kinase assay were measured by the methods of Labarca & Paigen (1980) and Wang & Smith (1975) respectively. The number of adipocytes/g of tissue in tissue pieces before culture was determined as described previously (Robertson *et al.*, 1982).

Statistical analysis

Results are expressed as means $\pm$ S.E.M., and were analysed by Student's *t* test for paired observations.

RESULTS

Pyruvate kinase

Preparatory experiments showed that maintenance of adipose tissue from sheep in culture in the absence or presence of insulin (17 nM) resulted in an increase in the maximum activity of pyruvate kinase (Table 1) and that this increase continued for at least 3 days (result not shown). The concentration of insulin required to achieve half-maximal increase was 85 pM, and less than 30% of the insulin added to the media was degraded over 48 h as found previously for bovine adipose tissue (Vernon *et al.*, 1985). The 15000 g-supernatant protein concentration of adipose-tissue pieces did not change over 48 h of tissue culture.

The ability of insulin to increase the activity of pyruvate kinase over 24 h and 48 h in tissue culture was not prevented by the addition of growth hormone (4.5 nM), prolactin (4 nM), glucagon (57 nM) or thyrotropin (80 pM) (results not shown). However, addition of cortisol (2.8 μ M) or theophylline (2 mM) prevented the increase in pyruvate kinase activity induced by insulin (Table 1). In this experiment the activity of pyruvate kinase was measured with 3 mM-phosphoenolpyruvate plus fructose 1,6-bisphosphate (to provide a measure of

Table 2. Effects of insulin and dexamethasone on pyruvate kinase activity of sheep adipose tissue

Adipose tissue from 3–4-year-old ewes was maintained in tissue culture for 24 or 48 h in the absence or presence of various combinations of insulin (170 pM), dexamethasone (10 nM), actinomycin D (80 nM) and antimycin A (36 μ M). Insulin and dexamethasone when added were present for the whole period of culture. Pyruvate kinase activity ($V_{\max}$) of adipose-tissue pieces was measured before and after tissue culture as described in the text. The activities presented are the changes in activity over either 0–24 h or 24–48 h of culture (a negative value means that the activity decreased over the period). All results are expressed per μ g of DNA of tissue pieces before tissue culture. The pyruvate kinase activity and the DNA concentrations of tissue before tissue culture were 6.7 ± 2.5 nmol/min per μ g of DNA and 63.7 ± 9.1 μ g of DNA/g wet wt. of tissue respectively (means $\pm$ S.E.M. for five results). Results are means $\pm$ S.E.M. for four observations (experiments involving antimycin A) or five observations (all others), and were analysed by Student's *t* test for paired observations. Comparisons: within rows, *, **, ***, value significantly different ($P < 0.05$, < 0.01 , < 0.001 respectively) from that obtained in the absence of insulin and dexamethasone, or, in the case of tissue exposed to insulin plus dexamethasone, from that obtained with insulin alone, over the same period; within columns, †, ††, †††, value significantly different ($P < 0.05$, < 0.01 , < 0.001 respectively) from that obtained in the absence of actinomycin D or antimycin A over the same period.

Period of change in activity	Additions to culture medium		Hormone ...	Pyruvate kinase: change in activity (nmol/min per μ g of DNA)			
	0–24 h	24–48 h		None	Insulin	Dexamethasone	Insulin + dexamethasone
0–24 h	None	–		4.2 ± 1.0	$10.7 \pm 3.3^*$	$1.4 \pm 1.0^{**}$	$3.7 \pm 1.0^*$
24–48 h	None	None		16.4 ± 2.9	$21.8 \pm 2.1^*$	$4.5 \pm 0.9^{**}$	$5.9 \pm 1.9^*$
0–24 h	Actinomycin D	–		$0.9 \pm 0.2^\dagger$	$1.5 \pm 1.0^\dagger$	0.8 ± 0.4	$0.7 \pm 0.6^\dagger$
24–48 h	Actinomycin D	Actinomycin D		$-2.8 \pm 0.6^{\dagger\dagger}$	$-3.2 \pm 1.1^{\dagger\dagger}$	$-2.4 \pm 1.0^{\dagger\dagger}$	$-1.8 \pm 0.5^{\dagger\dagger}$
24–48 h	None	Actinomycin D		12.3 ± 2.6	$16.8 \pm 1.4^*$	$3.9 \pm 1.0^{**}$	9.8 ± 2.3
0–24 h	Antimycin A	–		$2.4 \pm 1.3^\dagger$	$5.4 \pm 1.3^*$	$-0.5 \pm 0.5^*\dagger$	$1.5 \pm 0.5^*$
24–48 h	Antimycin A	Antimycin A		$9.7 \pm 1.6^\dagger$	$8.5 \pm 2.3^\dagger$	7.5 ± 1.9	6.3 ± 2.0

$V_{\max}$) and also with 0.05 mM-phosphoenolpyruvate in the absence of fructose 1,6-bisphosphate; in short-term incubations, insulin increases the ratio of pyruvate kinase activity measured at the two concentrations (activity ratio) in rat adipose tissue (Denton *et al.*, 1979). Incubation of sheep adipose tissue with insulin resulted in a transient increase in the activity ratio, with a maximum after about 6 h, after which it returned to the initial value after 24 h of incubation (Robertson *et al.*, 1982). In the present study the activity ratio declined over 48 h in culture, and this decrease was not affected by any of the hormones added (Table 1). Further studies therefore focused on the effect of insulin and glucocorticoids on the maximum activity of pyruvate kinase. The glucocorticoid analogue dexamethasone was used rather than cortisol, as the latter can be metabolized by adipose tissue (Weidenfeld *et al.*, 1982). Results in subsequent Tables are expressed as incremental increases over each 24 h period of study. However, the pattern of response of the $V_{\max}$ of pyruvate kinase to tissue culture in the absence and the presence of insulin over 24 h and 48 h were qualitatively identical and quantitatively very similar, both in the young male sheep used in the initial experiments and in the older ewes used in subsequent experiments described below. In addition, the effect of dexamethasone on the stimulation of pyruvate kinase activity by insulin was the same as that of cortisol.

The effects of insulin on pyruvate kinase activity were again apparent ($P < 0.05$) when adipose tissue was maintained in culture with a physiological concentration of insulin (170 pM) (Table 2). Again the increase in activity in the presence ($P < 0.05$) or absence ($P < 0.01$) of insulin over the second 24 h period (i.e. 24–48 h of culture) was significantly greater than during the first 24 h of culture. Inclusion of actinomycin D, an inhibitor

of transcription, in the culture media completely blocked the increase in pyruvate kinase over both periods in the presence ($P < 0.01$) or absence ($P < 0.01$) of insulin (Table 2). If actinomycin D was added after 24 h of culture (i.e. over the period 24–48 h), it had no effect on the increase in pyruvate kinase activity in the presence or absence of insulin.

Addition of 10 nM-dexamethasone to the culture markedly suppressed the increase in pyruvate kinase activity over both 24 h periods ($P < 0.01$), although an acceleration in increase in activity over the second 24 h was still apparent (Table 2). Inclusion of dexamethasone in the presence of insulin greatly decreased ($P < 0.01$) the effect of the latter hormone on pyruvate kinase. Dexamethasone had no effect on pyruvate kinase activity when actinomycin D was added at the start of the culture. When actinomycin D was added after the first 24 h of culture, it had no effect on the small increase in pyruvate kinase observed in the presence of dexamethasone alone or in the presence of dexamethasone plus insulin (Table 2).

Antimycin A is an inhibitor of the electron-transport chain, hence its presence renders cells effectively hypoxic and so should increase glycolysis. Addition of antimycin A to the culture media had no significant effect on the increase in pyruvate kinase activity over the first 24 h in the presence of insulin. During the second 24 h period, antimycin A blunted the increase in enzyme activity in the absence of hormones ($P < 0.05$) and in the presence of insulin alone ($P < 0.05$), while marginally (but not significantly) increasing the rise in activity in the presence of dexamethasone (Table 2); the overall effect of antimycin A was thus to minimize the differences in the increase in pyruvate kinase induced by the various hormones over the second 24 h of culture.

Table 3. Effects of insulin and dexamethasone on glucose utilization by sheep adipose tissue maintained in tissue culture

Adipose tissue was maintained in tissue culture for 24 or 48 h in the absence or presence of various agents as described in Table 2, and the amounts of glucose removed from the culture medium between 0 to 24 h and 0 to 48 h were measured. Further details are as in Table 2.

Period of utilization	Additions to culture medium		Hormone ...	Glucose utilization (nmol/24 h per μ g of DNA)			
	0-24 h	24-48 h		None	Insulin	Dexamethasone	Insulin + dexamethasone
0-24 h	None	—		184 $\pm$ 42	239 $\pm$ 56	112 $\pm$ 38***	176 $\pm$ 34
24-48 h	None	None		152 $\pm$ 49	281 $\pm$ 57**	9 $\pm$ 47***	-10 $\pm$ 67**
0-24 h	Actinomycin D	—		128 $\pm$ 21	142 $\pm$ 19†	147 $\pm$ 42†	156 $\pm$ 33
24-48 h	Actinomycin D	Actinomycin D		116 $\pm$ 21	125 $\pm$ 21†	71 $\pm$ 44†	98 $\pm$ 50††
24-48 h	None	Actinomycin D		170 $\pm$ 31	246 $\pm$ 75*	57 $\pm$ 50*††	103 $\pm$ 60***†
0-24 h	Antimycin A	—		260 $\pm$ 77†	297 $\pm$ 86*††	206 $\pm$ 82	235 $\pm$ 67*
24-48 h	Antimycin A	Antimycin A		330 $\pm$ 88††	472 $\pm$ 78***††	240 $\pm$ 56†††	462 $\pm$ 95††

In contrast with their effect on pyruvate kinase activity, insulin and dexamethasone acted in concert to increase the activity of glucose-6-phosphate dehydrogenase activity in adipose tissue from 3-4-year-old ewes. The activity of this enzyme was 162 ± 33 nmol/min per 10^6 cells before maintenance in culture; after 48 h in culture, the activity was 186 ± 50 , 213 ± 49 , 195 ± 62 and 300 ± 60 nmol/min per 10^6 cells for tissue maintained in the absence of added hormone, with 17 nM-insulin, with 10 nM-dexamethasone and with 17 nM-insulin plus 10 nM-dexamethasone (means $\pm$ S.E.M. for five observations). The value obtained with insulin plus dexamethasone was significantly ($P < 0.05$) greater than that found with insulin alone. In the same experiment dexamethasone again antagonized the stimulatory effects of insulin on pyruvate kinase activity (results not shown). The adipose tissue contained $1.3(\pm 0.16) \times 10^6$ adipocytes/g wet wt.

Adipose-tissue metabolism

In the absence of exogenous hormones, glucose uptake was similar over the first and second 24 h periods of culture (Table 3). Insulin (170 pM) increased ($P < 0.01$) glucose uptake, whereas dexamethasone resulted in a substantial decrease ($P < 0.001$) in uptake. Addition of actinomycin D had no significant effect on glucose uptake over either 24 h period in the absence of exogenous hormones. Addition of actinomycin D at the start of the culture inhibited glucose uptake over both 24 h periods in the presence of insulin ($P < 0.05$). In contrast, glucose uptake between 24 and 48 h in the presence of dexamethasone (with or without insulin) was enhanced ($P < 0.02$) by the presence of actinomycin D (Table 3). When actinomycin D was added after 24 h of culture, glucose uptake over the subsequent 24 h period was unaltered in the absence of exogenous hormones or in the presence of insulin alone (Table 3); however, uptake in the presence of dexamethasone alone or with insulin was increased ($P < 0.01$ and < 0.05 respectively).

Lactate is a major product of glycolysis in sheep adipose tissue (Khachadurian *et al.*, 1966; Robertson *et al.*, 1982). Lactate release accounted for about 25% of the glucose taken up over the first 24 h period in the presence or the absence of insulin, but only 14% in the

presence of dexamethasone (Table 4). Lactate output was significantly higher ($P < 0.05$) over the second 24 h period in the absence of hormones and in the presence of insulin alone. Dexamethasone decreased lactate output over both periods in the presence and the absence of insulin ($P < 0.01$). Actinomycin D resulted in a lower lactate output over the first 24 h in the absence of hormones ($P < 0.05$) and prevented the surge in lactate output in the presence and the absence of insulin during the second 24 h period ($P < 0.01$). Addition of actinomycin D after 24 h of culture, however, had no effect on lactate output in the absence of exogenous hormones or in the presence of insulin. In the presence of dexamethasone, actinomycin D had no effect on lactate output, except when added after 24 h to cultures containing insulin plus dexamethasone, when the inhibitor resulted in a higher rate of lactate output than in its absence ($P < 0.05$).

Antimycin A stimulated ($P < 0.01$) glucose uptake between 24 h and 48 h of culture in the presence and absence of the hormones (Table 3) and over the first 24 h in the absence of hormones ($P < 0.05$) and in the presence of insulin alone ($P < 0.01$). The effect of antimycin A was particularly marked in the presence of dexamethasone, and the agent completely overcame the antagonistic effects of dexamethasone on insulin-stimulated glucose uptakes over 48 h. Most of the additional glucose taken up in response to antimycin A was released as lactate; lactate output over both 24 h periods and in the presence and the absence of hormones was increased by antimycin A ($P < 0.05$) (Table 4).

Glycerol release into the culture medium provides a measure of basal lipolysis. In the absence of exogenous hormones, glycerol release was greater during the second 24 h period than the first ($P < 0.02$); insulin had no effect on its release, whereas dexamethasone resulted in a lower ($P < 0.05$) release over the first 24 h and suppressed the surge in release over the second 24 h period ($P < 0.01$) (Table 5). Actinomycin D had no significant effect over the first 24 h period, except in the presence of dexamethasone (with or without insulin), when it resulted in a higher ($P < 0.05$) rate of release. However, actinomycin D completely suppressed the increase ($P < 0.01$) in rate over the second 24 h period in the absence or presence of

Table 4. Effects of insulin and dexamethasone on net lactate release from sheep adipose tissue maintained in tissue culture

Adipose tissue was maintained in tissue culture for 24 or 48 h in the absence or presence of various agents as described in Table 2 and the net amount of lactate released into the culture medium was measured over 0–24 h and 0–48 h. Further details are as in Table 2.

Period of release	Additions to culture medium		Hormone ...	Net lactate release (nmol/24 h per μ g of DNA)			
	0–24 h	24–48 h		None	Insulin	Dexamethasone	Insulin + dexamethasone
0–24 h	None	–		100 $\pm$ 12	115 $\pm$ 10	32 $\pm$ 5**	51 $\pm$ 9***
24–48 h	None	None		172 $\pm$ 56	304 $\pm$ 62*	50 $\pm$ 30**	30 $\pm$ 28***
0–24 h	Actinomycin D	–		48 $\pm$ 10†	49 $\pm$ 15†	32 $\pm$ 17	47 $\pm$ 17
24–48 h	Actinomycin D	Actinomycin D		55 $\pm$ 32†	51 $\pm$ 31††	62 $\pm$ 41	63 $\pm$ 21
24–48 h	None	Actinomycin D		152 $\pm$ 42	227 $\pm$ 54*	81 $\pm$ 27*	131 $\pm$ 33*†
0–24 h	Antimycin A	–		270 $\pm$ 53†	319 $\pm$ 71†	222 $\pm$ 60†	282 $\pm$ 65†
24–48 h	Antimycin A	Antimycin A		526 $\pm$ 85†	643 $\pm$ 151†	300 $\pm$ 58**†	628 $\pm$ 153†

Table 5. Effects of insulin and dexamethasone on glycerol release from sheep adipose tissue maintained in tissue culture

Adipose tissue was maintained in tissue culture for 24 or 48 h in the absence or presence of various agents as described in Table 2 and the amount of glycerol released into the culture medium over 0–24 h and 0–48 h was measured. Further details are as in Table 2.

Period of release	Additions to culture medium		Hormone ...	Net glycerol release (nmol/24 h per μ g of DNA)			
	0–24 h	24–48 h		None	Insulin	Dexamethasone	Insulin + dexamethasone
0–24 h	None	–		15.0 $\pm$ 3.5	13.4 $\pm$ 3.1	6.6 $\pm$ 1.6*	8.2 $\pm$ 1.6*
24–48 h	None	None		36.6 $\pm$ 6.4	38.0 $\pm$ 9.1	7.4 $\pm$ 3.6**	4.4 $\pm$ 3.4**
0–24 h	Actinomycin D	–		11.6 $\pm$ 3.3	12.0 $\pm$ 2.9	10.2 $\pm$ 1.6††	10.2 $\pm$ 2.1†
24–48 h	Actinomycin D	Actinomycin D		11.0 $\pm$ 2.7††	6.2 $\pm$ 2.6*††	5.0 $\pm$ 2.6*	8.0 $\pm$ 2.7†
24–48 h	None	Actinomycin D		22.2 $\pm$ 3.9	28.0 $\pm$ 5.6	8.2 $\pm$ 3.6**	6.2 $\pm$ 4.0**
0–24 h	Antimycin A	–		11.3 $\pm$ 3.1	11.8 $\pm$ 2.0	8.8 $\pm$ 2.3	10.5 $\pm$ 1.4
24–48 h	Antimycin A	Antimycin A		22.1 $\pm$ 2.6†	24.9 $\pm$ 4.0†	15.9 $\pm$ 2.3*†	21.8 $\pm$ 0.5†

insulin when given at the start of the culture. When added after 24 h, actinomycin D prevented the increase in glycerol release in the absence of exogenous hormones. Antimycin A had no effect on glycerol release over the first 24 h period, but it diminished the rate of release over the second 24 h period in the absence of hormones and in the presence of insulin alone ($P < 0.05$), while increasing ($P < 0.05$) the rate of glycerol release in the presence of dexamethasone with and without insulin (Table 5).

DISCUSSION

Although the primary objective of the present study was to provide further insight into the long-term regulation of pyruvate kinase of adipose tissue, the study also provides further information on the control of adipose-tissue metabolism by insulin and glucocorticoids in sheep.

Pyruvate kinase

The present study confirms and extends previous observations that there are differences in the control of

the pyruvate kinase isoenzyme activity of adipose tissue and hepatocytes, although the data for hepatocytes are for rats (there are no comparable results for sheep hepatocytes), hence a possible species rather than tissue difference cannot be excluded as yet. Thus, when maintained in culture in the absence of exogenous hormones, pyruvate kinase activity declines in rat hepatocytes (Miyanaga *et al.*, 1982, 1983; Evans *et al.*, 1984; Fleig *et al.*, 1984), whereas it increases in adipose tissue from man (Smith *et al.*, 1976) and cattle (Vernon *et al.*, 1985). In addition, although glucocorticoids act in concert with insulin to maintain pyruvate kinase in rat hepatocytes maintained in culture (Feliú *et al.*, 1982; Miyanaga *et al.*, 1982, 1983; Evans *et al.*, 1984; Fleig *et al.*, 1984), we now show that they antagonize the effects of insulin on the enzyme activity in sheep adipose tissue. Furthermore, the effects of insulin on pyruvate kinase activity in adipose tissue were prevented by an inhibitor of transcription, suggesting that the hormone increases enzyme synthesis, whereas in rat hepatocytes in culture the effects of insulin may be due primarily to inhibition of pyruvate kinase degradation (Poole & Bloxham, 1982). However, insulin does stimulate synthesis of

pyruvate kinase in hepatocytes from diabetic (Miyanaga *et al.*, 1982) and starved (Evans *et al.*, 1984) rats. There are also some similarities: insulin alone increases the activity (or at least limits the decline) of pyruvate kinase, whereas glucocorticoids in the absence of insulin decrease the activity of the enzyme *in vitro* in rat hepatocytes (Miyanaga *et al.*, 1982, 1983; Fleig *et al.*, 1984) and in sheep adipose tissue (Tables 1 and 2). In addition, insulin neither alone nor in combination with a variety of other hormones could maintain the activity ratio of the enzyme in either adipose tissue (Table 1) or in rat hepatocytes maintained in culture (Fleig *et al.*, 1984).

The differences between rat hepatocytes and sheep adipose tissue in the control of pyruvate kinase activity are in marked contrast with the control of glucose-6-phosphate dehydrogenase, the activity of which is enhanced synergistically in both rat hepatocytes (Kurtz & Wells, 1981) and sheep adipose tissue (the present study) by insulin and dexamethasone. Furthermore, insulin and glucocorticoids act in concert *in vitro* to promote lipogenesis in adipose tissue from sheep (Vernon & Finley, 1986), pigs (Walton *et al.*, 1986) and cattle (Etherton & Evoke, 1986) and in hepatocytes from rats (Amatruda *et al.*, 1983). The effect of glucocorticoids on insulin stimulation of lipogenesis in rat adipose tissue *in vitro* is not clear, with reports of antagonism (Philipps *et al.*, 1965), synergism (Minshull & Strong, 1985) and no effect (Hallowes *et al.*, 1973), but *in vivo* they are antagonistic (see Fain, 1979). These species differences may arise from glucose rather than acetate being the major lipogenic precursor in rats. However, insulin and glucocorticoids act synergistically to increase lipoprotein lipase activity of rat adipose tissue (Ashby & Robinson, 1980; Speake *et al.*, 1986). Hence the antagonistic effects of glucocorticoids on insulin-stimulated pyruvate kinase activity in sheep adipose tissue appear to be specific and not the result of a general decrease in enzyme synthesis.

A previous study (Robertson *et al.*, 1982) showed that the increase in pyruvate kinase activity in adipose tissue during culture paralleled an increase in glycolytic rate. Studies with cell lines have shown that hypoxic conditions increased both the rate of glycolysis and the activity of glycolytic enzymes, including pyruvate kinase (Robin *et al.*, 1984; Ptashne *et al.*, 1985). Antimycin A in the present study also appeared to increase glycolysis, as evidenced by the increase in glucose uptake and lactate output in its presence, but failed to increase pyruvate kinase activity (at least in the absence of dexamethasone), suggesting that changes in pyruvate kinase in sheep adipose tissue are not directly linked to changes in glycolytic flux.

Adipose-tissue metabolism

As described above, adipose tissue from sheep and cattle shows little or no response to insulin during short-term incubations, but shows a clear response to physiological concentrations of the hormone during incubations for 24 h or longer (Vernon, 1982; Vernon *et al.*, 1985; Etherton & Evoke, 1986). The mechanism underlying this longer-term effect of insulin is unknown, but the present study clearly indicates the involvement of transcription and suggests that production of a peptide intermediate in insulin action is a requisite. The lack of an inhibitory effect of actinomycin D when added after 24 h suggests that sufficient of this putative intermediate is produced during the first 24 h to permit response to

insulin during the following 24 h period. Exposure of rat adipose tissue to insulin *in vivo* also increases the responsiveness of the tissue to insulin (Livingston & Moxley, 1982; James *et al.*, 1985), but the mechanism is unknown.

Dexamethasone is known to impair glucose utilization, probably by inhibition of glucose translocase activity in the absence of insulin and also in the presence of insulin at glucose concentrations below 1 mM (when glucose transport is limiting) in rat adipocytes by a process involving protein synthesis (see Fain, 1979). The results of the present study show that a similar but not identical phenomenon pertains in sheep. In contrast with rat adipose tissue, dexamethasone inhibited insulin-stimulated glucose utilization in sheep adipose tissue at glucose concentrations above 1 mM. This suggests that, whereas glucose transport is probably the primary site of glucocorticoid antagonism in rat adipocytes, this would not appear to be the case in sheep adipose tissue. This view is supported by the effects of antimycin A, which markedly stimulated glucose utilization in sheep adipose tissue even in the presence of dexamethasone, and in fact completely overcame the effects of dexamethasone on insulin-stimulated glucose utilization. As noted above, antimycin A inhibits the respiratory chain and so increases the need to produce ATP by glycolysis. As glucose uptake and release as lactate increased in response to an increased requirement for glycolysis, even in the presence of dexamethasone, it follows that the primary site of dexamethasone cannot be at the level of glucose transport in sheep adipose tissue. Rather, it appears that dexamethasone decreases the need for glucose metabolism in sheep adipose tissue, perhaps by decreasing the need for ATP production by glycolysis. This could be via a decrease in the ATP requirement of the cell or by increasing the efficiency of oxidative phosphorylation. Another possibility, that dexamethasone promotes fatty acid oxidation, seems unlikely, as it decreases lipolysis and, in the presence of insulin, enhances fatty acid synthesis (see above). Thus, although glucocorticoids have similar antagonistic effects on glucose metabolism in adipose tissue from sheep and rats, the sites of action show major differences.

Glycerol release during tissue culture was also decreased by the presence of dexamethasone. This was unexpected, for, in the rat (see Fain, 1979) and man (Cigolini & Smith, 1979), glucocorticoids do not alter the rate of glycerol release from adipose tissue themselves during short-term incubations (up to 4 h), although they do have permissive effects with other hormones and increase the rate of fatty acid release by inhibiting re-esterification (see Leung & Munck, 1975; Fain, 1979). This does not appear to be a species difference, for we have found that the presence of dexamethasone results in a lower rate of glycerol release from rat adipose tissue maintained in culture for 24 h (results not shown). Also, tissue culture of human adipose tissue for several days in the presence of cortisol tended to decrease the rate of glycerol release, although this could have been due to a decrease in cell size (Cigolini & Smith, 1979). The mechanism responsible for the inhibition of lipolysis by dexamethasone is unclear. However, one possibility, a decrease in re-esterification with a concomitant increase in intracellular fatty acids, seems unlikely, for insulin did not relieve the inhibition of lipolysis, and, as noted above, insulin and dexamethasone act synergistically to

increase fatty acid synthesis and activate acetyl-CoA carboxylase (E. Finley and R. G. Vernon, unpublished work), which is inconsistent with a rise in the intracellular fatty acid concentration.

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REFERENCES

- Amatruda, J. M., Danahy, S. A. & Chang, C. L. (1983) *Biochem. J.* **212**, 135–141
- Ashby, P. & Robinson, D. S. (1980) *Biochem. J.* **188**, 185–192
- Ballard, F. J., Hanson, R. W. & Kronfeld, D. S. (1969) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **28**, 218–231
- Cigolini, M. & Smith, U. (1979) *Metab. Clin. Exp.* **28**, 502–510
- Cimbala, M. A., Lau, D. & Daigneault, J. F. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., ed.), pp. 385–395, Plenum Press, London
- Claus, T. H. & Pilkis, S. J. (1981) *Biochem. Actions Horm.* **8**, 209–271
- Denton, R. M. & Halestrap, A. P. (1979) *Essays Biochem.* **15**, 37–77
- Denton, R. M., Edgell, N. J., Bridges, B. J. & Poole, G. P. (1979) *Biochem. J.* **180**, 523–531
- Engström, L. (1980) in *Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation* (Cohen, P., ed.), pp. 11–31, Elsevier, Amsterdam
- Etherton, T. D. & Evoke, C. M. (1986) *J. Anim. Sci.* **62**, 357–362
- Evans, C., Miyanaga, O. & Cottam, G. L. (1984) *Arch. Biochem. Biophys.* **233**, 617–623
- Fain, J. N. (1979) in *Glucocorticoid Hormone Action* (Baxter, J. D. & Rousseau, G. G., eds.), pp. 547–560, Springer-Verlag, Berlin
- Feliú, J. E., Coloma, J., Gómez-Lechón, M.-J., García, M. D. & Báguena, J. (1982) *Mol. Cell. Biochem.* **45**, 73–81
- Fleig, W. E., Geerling, I., Röben, H. & Ditschuneit, H. (1984) *Biochim. Biophys. Acta* **805**, 165–173
- Hallowes, R. C., Wang, D. Y., Lewis, D. J., Strong, C. R. & Dils, R. (1973) *J. Endocrinol.* **57**, 265–276
- James, D. E., Burleigh, K. M. & Kraegen, E. W. (1985) *Diabetes* **34**, 1049–1054
- Khachadurian, A. K., Adrouni, B. & Yacoubian, H. (1966) *J. Lipid Res.* **7**, 427–436
- Kurtz, J. W. & Wells, W. W. (1981) *J. Biol. Chem.* **256**, 10870–10875
- Labarca, C. & Paigen, K. (1980) *Anal. Biochem.* **102**, 344–352
- Leung, K. & Munck, M. (1975) *Annu. Rev. Physiol.* **37**, 245–272
- Livingston, J. N. & Moxley, R. T. (1982) *Endocrinology* (Baltimore) **111**, 1749–1751
- Minshull, M. & Strong, C. R. (1985) *Int. J. Biochem.* **17**, 529–532
- Miyanaga, O., Nagano, M. & Cottam, G. L. (1982) *J. Biol. Chem.* **257**, 10617–10623
- Miyanaga, O., Evans, C. & Cottam, G. L. (1983) *Biochim. Biophys. Acta* **758**, 42–48
- Philipps, G. R., Herrera, M. G. & Renold, A. E. (1965) *Biochim. Biophys. Acta* **106**, 234–240
- Poole, G. P. & Bloxham, D. P. (1982) *Biochem. J.* **204**, 89–95
- Ptashne, K. A., Morin, M. E., Hance, A. & Robin, E. D. (1985) *Biochim. Biophys. Acta* **844**, 19–23
- Robertson, J. P., Faulkner, A. & Vernon, R. G. (1982) *Biochem. J.* **206**, 577–586
- Robin, E. D., Murphy, B. J. & Theodore, J. (1984) *J. Cell. Physiol.* **11**, 287–290
- Smith, U., Boström, S., Johansson, R. & Nyberg, G. (1976) *Diabetologia* **12**, 137–143
- Speake, B. K., Parkin, S. M. & Robinson, D. S. (1986) *Biochim. Biophys. Acta* **881**, 155–157
- Vernon, R. G. (1980) *Prog. Lipid Res.* **19**, 23–106
- Vernon, R. G. (1982) *Int. J. Biochem.* **14**, 255–258
- Vernon, R. G. & Finley, E. (1986) *Biochem. Soc. Trans.* **14**, 635–636
- Vernon, R. G., Clegg, R. A. & Flint, D. J. (1981) *Biochem. J.* **200**, 307–314
- Vernon, R. G., Finley, E., Taylor, E. & Flint, D. J. (1985) *Endocrinology* (Baltimore) **116**, 1195–1199
- Walton, P. E., Etherton, T. D. & Evoke, C. M. (1986) *Endocrinology* (Baltimore) **118**, 2577–2581
- Wang, C. S. & Smith, R. L. (1975) *Anal. Biochem.* **63**, 414–417
- Weidenfeld, J., Siegel, R. A., Levy, J. & Chowder, I. (1982) *J. Steroid Biochem.* **17**, 357–360
- Yang, Y. T. & Baldwin, R. L. (1973) *J. Dairy Sci.* **56**, 350–365

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