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Long term culture of rat soleus muscle in vitro

Its effects on glucose utilization and insulin sensitivity

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Rat soleus muscle strips cultured for 24 h in medium 199 were well preserved in terms of electron microscopy; ATP and creatine phosphate concentrations; rates of glucose utilization, glycogen and protein synthesis, and effects of insulin thereon. Culture led to modest changes in fluid spaces and intracellular (K^+); increased basal glucose utilization up to two-fold; had no effect on the maximum response to insulin; and had no effect on sensitivity to insulin except in the presence of adenosine deaminase. Thus in vitro neither denervation nor absence of insulin had any marked effects in 24 h to decrease responses to insulin.

Muscle culture; Muscle viability; Glucose utilization; Insulin sensitivity; Adenosine; Denervation

1. INTRODUCTION

Longer term mechanisms (hours to days) are important in the regulation of carbohydrate metabolism in animals and obvious examples include in vivo effects in muscles of dietary variation or diabetes on glucose utilization and oxidation [1,2]; on insulin sensitivity [3,4]; on the activities of pyruvate dehydrogenase (PDH) complex and PDH kinase in muscles and liver [5]; and on the activities of key enzymes of glucose utilization and gluconeogenesis in liver [6]. Culture in chemically defined media has provided key information about hormones and nutrients that mediate longer term effects of diet and diabetes on carbohydrate metabolism in hepatocytes and cardiac myocytes [5-7]. An equivalent culture system for skeletal muscle is urgently needed as it is the major site of glucose utilization in animals. Survival of rat epitrochlearis muscle for 30 h in the presence of rat serum has been described [3] but in the absence of serum, survival of cultured epitrochlearis and soleus muscles was limited to 12 h duration [3,9]. A longer period of survival is required, and absence of serum is essential because serum is not chemically defined. We describe here a method that has yielded consistently, apparently satisfactory survival of rat soleus muscle in vitro for at least 24 h in the absence of serum (the longest period tested). We show for the first time the in vitro effects of 24 h of total deficiency of added hormones and denervation on rates of glucose utilization and insulin sensitivity in soleus muscle.

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2. EXPERIMENTAL

Sources of rats and details of feeding were as in [7]. Biochemicals were from Sigma Chemical Co. or BCL, medium 199 from Gibco, radiochemicals from Amersham and Optifluor from Canberra Packard.

Strips (<40 mg) from soleus muscles of anaesthetised rats of 160-180 g were mounted on stainless steel clips (Crettaz et al. [10]) and suspended in medium 199 by cotton threads so as not to touch the flask. Culture was for up to 24 h at 37°C in 50 ml conical flasks, in 30 ml medium 199 supplemented with streptomycin and penicillin [8], and pre-equilibrated with O_2/CO_2 (95:5). Flasks were shaken at 110 cycles/min and re-gassed every 3 h. For analysis of metabolites, powdered frozen muscle (liquid N_2) was extracted with 0.6 M perchloric acid, and extracts neutralised with saturated $KHCO_3$. ATP and creatine phosphate were assayed with hexokinase, glucose-6-phosphate dehydrogenase and creatine kinase [11,12]; and glucose (and glycogen glucose after hydrolysis with *A. niger* amyloglucosidase) with hexokinase, glucose-6-phosphate dehydrogenase and ATP [13]. Recoveries of ATP and creatine phosphate added to frozen muscle before extraction were >90%. Lactate dehydrogenase was estimated in incubation medium and in polytron extracts of muscle strips [14]. Total water was estimated by dry weight analysis. Inulin and glucose spaces were estimated in strips incubated for 30 min with [^{3}H]inulin and 5.5 mM glucose and extracted by boiling. In control experiments 30 min of incubation sufficed to equilibrate muscle with extracellular inulin and glucose (results not shown). Inulin was assayed by liquid scintillation spectrometry in Optifluor with quench corrections by external standard; glucose was assayed as above. Soleus K^+ was assayed in concentrated HNO_3 digests by flame photometry against KCl standards; corrections for extracellular K^+ were based on inulin space. Glucose utilization was measured by formation of [^{3}H]H_2O from 2-[^{3}H]glucose; [^{3}H]H_2O was separated from 2-[^{3}H]glucose by vacuum distillation [15]. Amino acid incorporation into protein was measured as in [16] after incubating muscle strips for 2 h in medium 199 containing U-[^{14}C]amino acid mixture (4.7×10^5 dpm/ml).

3. RESULTS AND DISCUSSION

Medium 199 contains as potential respiratory

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substrates glucose (5.5 mM), acetate (0.6 mM) and a full complement of amino acids (for full composition see [8]). Acetate is utilized by soleus muscle and inhibits glycolytic flux 30% at 1.2 mM [10]. Culture (24 h) of rat soleus had no effect on concentrations of ATP and creatine phosphate; or rates of amino acid incorporation into protein; or basal rates of glucose utilization or stimulation by insulin (10 mU/ml) (Table I). Insulin had no significant effect on amino acid incorporation into protein in fresh muscle but it stimulated incorporation in cultured muscle. The absence of the insulin effect in fresh muscle may be related to the well known stimulation of amino acid incorporation by leucine, present in medium 199 and which may override the effect of insulin in short term incubations [17]. The effect of longer term culture has not previously been amenable to investigation. Culture (24 h) decreased intracellular K^+ concentration (19%) and increased total water (3%), and inulin space (24%) but had no effect on muscle glucose space. Glucose and inulin spaces did not differ significantly (Table I). Soleus muscle lactate dehydrogenase (LDH) activity was not changed significantly by culture (24 h), but LDH equivalent to 12% of the muscle activity was released into the culture medium within the first 8 h of incubation, but not thereafter (Fig. 1). Muscle glycogen concentration fell by 40% over 24 h of culture (Table I); the decrease is virtually identical with that induced in vivo by 24 h of starvation [18]. The decrease in glycogen concentration was largely reversed by 1 h of incubation with insulin (1 mU/ml) in vitro. The effect of insulin on glycogen synthesis in fresh muscle was quantitatively similar to that

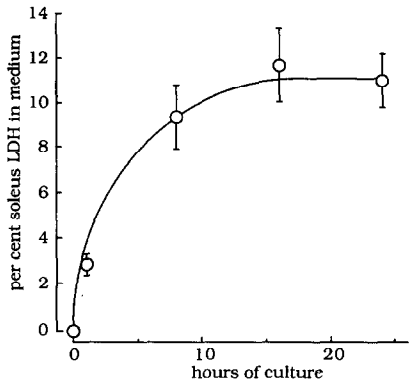

Fig. 1. Appearance of lactate dehydrogenase activity in medium 199 during 24 h of culture of rat soleus muscle strips (mean ± SE for 14 strips).

in [10] but was not statistically significant in our experiment due to a greater SE when concentration differences are measured. Electron microscopy showed no systematic differences between fresh and cultured muscles, although occasional individual fibres showed evidence of gross damage or of damage to mitochondria (results not shown).

Concentrations of ATP, glycogen, and K^+ and rates of glucose utilization in the present study were comparable to those obtained with fresh soleus strips in other studies [10,19,20]. Glucose was apparently confined to extracellular water, i.e. utilization was apparently limited by the rate of glucose transport. The concentration of creatine phosphate and the ratio of (creatine phosphate)/(ATP) was somewhat lower than

Table I

Effect of in vitro culture (24 h) on concentrations of metabolites and ions, fluid spaces, and rates of glucose utilization, glycogen synthesis and amino acid incorporation into protein in rat soleus muscle strips

Measurement	Insulin (mU/ml)	Fresh muscle	Muscle cultured 24 h
ATP (μmol/g fresh wt)	-	4.69 ± 0.26 (15)	4.72 ± 0.26 (48)
Creatine phosphate (μmol/g fresh wt)	-	6.32 ± 0.52 (15)	6.13 ± 0.44 (48)
Lactate dehydrogenase (U/g fresh wt)	-	141 ± 10.7 (8)	132 ± 3.99 (8)
Total water (μl/g fresh wt)		790 ± 4.7 (8)	811 ± 5.1[b] (8)
Inulin space (μl/g fresh wt)		222 ± 9.8 (14)	275 ± 16.4[c] (14)
Glucose space (μl/g fresh wt)		244 ± 24 (6)	242 ± 24.2 (6)
Intracellular K^+ (μmol/g fresh wt)	-	78.3 ± 1.65 (8)	63.6 ± 2.45[a] (8)
(μmol/ml intracellular water)		139 ± 3.3 (8)	117 ± 4.06[a] (8)
Amino acid incorporation into protein			
(dpm per mg protein/2 h):-	-	1609 ± 122 (8)	1679 ± 126 (12)
	10	1661 ± 66 (8)	2025 ± 112[d] (8)
Muscle glycogen concentration	-	14.7 ± 0.5 (18)	9.5 ± 0.44[a] (16)
(μmol glycogen glucose/g fresh wt)			
	1[e]	16.9 ± 1.9 (14)	13.2 ± 0.39[d] (8)
Difference (insulin effect)		2.2 ± 2	3.7 ± 0.59[d]
Glucose utilization	-	5.86 ± 0.90 (6)	4.76 ± 0.20 (6)
(μmol/g fresh wt/h)	10	12.66 ± 0.42[d] (6)	10.36 ± 0.34[a,d] (6)
Difference (insulin effect)		6.80 ± 0.99	5.60 ± 0.39[d]

Glucose utilization measured from 1–2 h of incubation (fresh muscle) or from 24–25 h of incubation (cultured muscle); amino acid incorporation was measured from 0–2 h (fresh muscle) or from 24–26 h (cultured muscle); [e]insulin present from 30–90 min (fresh) or 24–25 h (cultured); glycogen values at 90 min or 25 h. [a]$P<0.001$, [b]$P<0.01$, [c]$P<0.02$ for effect of culture; [d]$P<0.01$ for effect of insulin: for other differences $P>0.05$: the number of soleus muscles strips is shown in parentheses.

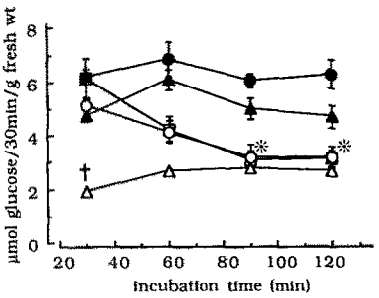

Fig. 2. Progressive rates of glucose utilization of six rat soleus muscle strips, measured by the rate of formation of $[^3H]H_2O$ from [2-^{3}H]glucose during four successive 30 min periods. The muscle strips were transferred to fresh medium at the beginning of each 30 min period. Results are means ± SE. Open symbols, no insulin; closed symbols, insulin (10 mU/ml); □ adenosine deaminase 2 U/ml; ○ □ -fresh muscle strips; ▲ △ -muscle strips cultured for 24 h before incubation with [2-^{3}H]glucose. $P<0.01$ for effect of insulin at all periods of incubation except fresh muscle 0–30 min; $^*P<0.01$ for effect of incubation time; $^\dagger P<0.01$ for effect of culture.

in [10,20] though recoveries of added creatine phosphate were >90% (see section 2). Extracellular water was greater than in vivo (140 μl/g [21]); this was accounted for in fresh muscle by a 10% increase in total water within 60 min in vitro. The further increase after 24 h could be an effect of denervation [21].

We conclude that soleus muscle strips remained viable for at least 24 h (the longest period tested) under the conditions of culture which we have defined. The only obvious potential basis for extended viability in the present study (cf. [3,9]) is our use of medium 199, but the nature of the components responsible (e.g. whether nutrients or antioxidants) has not been determined.

Fig. 2 shows rates of glucose utilization measured during four successive 30 min periods in fresh muscles and in muscles cultured for 24 h. Rates with insulin (10 mU/ml) showed no significant variation, either during successive 30 min periods or as between fresh and cultured muscles. In the absence of insulin glucose utilization by fresh muscle declined over 60–90 min to a minimum rate which was maintained over 24 h of culture (assumed to be due to loss of the action of insulin carried over from in vivo). Adenosine deaminase which sensitises soleus muscle to insulin (see below) had no effect on this decline but may have enhanced glucose utilization during the first 30 min period, possibly through an effect on insulin sensitivity (see below). In subsequent studies, rates of glucose utilization in fresh muscles were measured between 60 and 90 min of incubation, i.e. when the basal rate was steady state and at a minimum.

Table II and Fig. 3 show the effects of 24 h of culture, and of an adenosine antagonist (phenyltheophylline (PT), 2 μM), or adenosine deaminase (0.5 U/ml) on basal and insulin stimulated rates of glucose utilization. In the absence of PT or adenosine deaminase, culture of soleus increased basal glucose utilization by 30% in the experiment in Table II but had no significant effect in an earlier experiment in Table I in which SE were larger. Culture did not significantly affect sensitivity to stimulation by insulin (not detected below 625 μU/ml). The effect of culture to increase basal glucose utilization was markedly enhanced (to up to 100%) by PT and by adenosine deaminase - due mainly to their effect on cultured muscle. The effect of culture on basal glucose utilization was only partly reversed (<25%) by inclu-

Table II

Effect of culture (24 h), of phenyltheophylline, and of adenosine deaminase on basal and insulin stimulated rates of glucose utilization in rat soleus muscle strips

Muscle	Insulin (μU/ml)	Glucose utilization (μmol/g muscle/30 min) mean ± SE		
		Control	Phenyltheophylline (2 μM)	Adenosine deaminase (0.5 U/ml)
Fresh	–	1.58 ± 0.14	1.45 ± 0.27	2.00 ± 0.14
24 h culture	–	2.10 ± 0.08[e]	2.88 ± 0.20[c,e]	3.55 ± 0.29[c,e]
Fresh	25	1.99 ± 0.22	1.83 ± 0.24	2.89 ± 0.21[a]
24 h culture	25	2.25 ± 0.20	3.59 ± 0.06[b,d,e]	3.99 ± 0.23[c,e]
Fresh	125	1.99 ± 0.16	3.45 ± 0.63[b]	3.64 ± 0.35[a]
24 h culture	125	2.48 ± 0.27	4.75 ± 0.80[c]	4.08 ± 0.31[c]
Fresh	625	3.14 ± 0.14[a]	3.70 ± 0.32[a]	4.76 ± 0.35[a]
24 h culture	625	3.30 ± 0.23[a]	5.15 ± 0.35[a,c,f]	4.61 ± 0.20[a,c]
(number of strips)		(12)	(4)	(8) (fresh) (12) (24 h culture)

Glucose utilization measured during 60–90 min of incubation (fresh muscles) or 24.5–25 h of incubation (cultured muscles) by $[^3H]H_2O$ production from [2-^{3}H]glucose added at 60 min or 24.5 h. Other additions were made at 30 min or 24 h (insulin) or zero min or 23.5 h (phenyltheophylline or adenosine deaminase). $^aP<0.01$, $^bP<0.05$ for effect of insulin; $^cP<0.01$, $^dP<0.05$ for effect of phynyltheophylline or adenosine deaminase; $^eP<0.01$, $^fP<0.05$ for effect of culture.

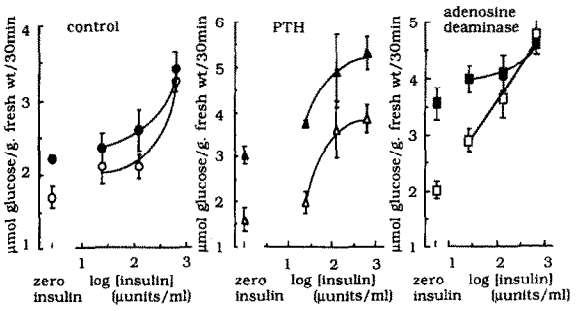

Fig. 3. Effect of insulin at 25, 125 and 625 μU/ml on rates of glucose utilisation by rat soleus muscle strips. Each point is mean ± SE for data taken from Table II. Open symbols are fresh muscles; closed symbols are muscles cultured for 24 h in medium 199. PTH - phenyltheophylline.

sion of insulin (10 mU/ml) or adenosine deaminase throughout (results not shown).

As shown by Espinal et al. [22] sensitivity to insulin in fresh muscle was increased by PT or adenosine deaminase (down to 125 and 25 μU/ml respectively). In cultured muscle sensitivity to insulin was increased 25-fold by PT (down to 25 μU/ml) but not by adenosine deaminase (insulin response at 625 μU/ml only). The higher basal rates of glucose utilization in cultured muscle in the presence of adenosine deaminase may have obscured the insulin response at lower concentrations of the hormone. Except at the highest insulin concentration (625 μU/ml) rates of glucose utilization in cultured muscles were greater than in fresh muscles.

Two factors which could contribute to effects of 24 h of culture on metabolic parameters are absence of hormones and denervation. The increase in basal glucose utilization observed in the present study may be an effect of denervation as a comparable effect has been described in rat diaphragm after denervation [23,24]. The in vivo effect of denervation (24 h) to decrease substantially insulin-induced glycogen synthesis in soleus [25] and diaphragm [24] was not seen in vitro in the present study. Factors in addition to denervation per se may therefore be involved in vivo. Denervation and absence of added insulin were without effects on basal protein synthesis and enhanced the effect of insulin in vitro in the present study [cf. 21,26]); likewise there was no evidence in the present study for any marked in vitro effect of 24 h of denervation and absence of insulin to diminish the response of soleus muscle to insulin effects of glucose utilization (cf [21,23-27]).

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