Biochem. J. (1990) 267, 257–260 (Printed in Great Britain)

Use of the intact mouse skeletal-muscle preparation for metabolic studies

Evaluation of the model

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1. We examined the isolated mouse skeletal-muscle model *in vitro*, commonly used by many investigators, for its suitability for metabolic studies. 2. Despite the fact that pH, O_2 saturation, osmolality and the release of the enzyme creatine kinase remained stable, histochemical studies showed large cores devoid of glycogen, suggesting that the incubated muscle had lost its viability. 3. This study indicates that caution should be exercised when interpreting the results of studies with intact isolated mouse muscles.

INTRODUCTION

It is well recognized that skeletal-muscle metabolism is under strict control of the endocrine system [1,2]. Unlike the situation in the intact animal, the isolated muscle model makes it possible to study particular effects of hormones, uncomplicated by side effects of other substances that might occur *in vivo*. However, this model *in vitro* does not optimally reflect the situation *in vivo*. A major disadvantage of the model might be the absence of capillary flow, which could severely hamper substrate and O_2 diffusion into muscle cells. It has been shown that incubation of skeletal muscles of young rats (weighing 40–70 g) *in vitro* inevitably led to core formation in which loss of glycogen occurred [3]. Several investigators [4–11] assumed (but did not provide firm evidence) that mouse and other small-rodent skeletal muscles are thin enough to avoid diffusion problems into the inner part of the muscle. However, core formation and diffusion of O_2 are influenced, not only by the tissue thickness, but also by the metabolic rate of the donor animal [3,12] and the temperature of the buffer solution. Therefore the aim of the present study was to evaluate the mouse skeletal-muscle preparation *in vitro* as described by Le Marchand-Brustel *et al.* [7] and Bonen *et al.* [4] in order (*a*) to determine whether we could reproduce the results of the aforementioned investigators, (*b*), if so, to determine what accounted for the enormous variation, and (*c*) to evaluate the viability of the incubated muscles by employing histochemical techniques.

MATERIALS AND METHODS

Fiftyfour male Swiss–Webster mice weighing 30–32 g were obtained from Charles River Wiga G.m.b.H. (Sulzfeld, Germany). The animals were housed in groups of eight in a constant-temperature (22 ± 1 °C) room on a 12 h-light (07:30–19:30 h)/12 h-dark (19:30–07:30 h) cycle and had free access to water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Procedures were performed as described by Le Marchand-Brustel *et al.* [7] and Bonen *et al.* [4]. Briefly, at the time of the experiment the mice were anaesthetized with phentanyl/fluanisone (0.05 ml/g body wt., subcutaneously) (Hypnorm; Janssen, Beerse, Belgium). From the hindlimbs both the soleus and the extensor digitorum longus muscles were carefully removed, weighed on a Mettler balance and mounted on a stainless-steel V-shaped clip. Each muscle was individually immersed at 0 °C in an oxygenated (O_2/CO_2, 19:1) Krebs–Ringer bicarbonate buffer, pH 7.4 (Bonen *et al.* [4]), containing 4% (w/v) BSA (essentially fatty acid-free, catalogue no. A-6003; Sigma Chemical Co., St. Louis, MO, U.S.A.). After the muscles of one animal were dissected free, requiring about 15 min, they were transferred to plastic vials (Poly Q; Beckman, Galway, Ireland) containing 1.5 ml of Krebs–Ringer bicarbonate buffer supplemented with 2 mM-pyruvate and either 0, 0.4, 1.0 or 10.0 munits of insulin (pig insulin, catalogue no. I-3505; Sigma Chemical Co.)/ml. The muscles were randomized so that, of each pair from one animal, one was used as test muscle and the contralateral as control muscle. All the muscles were then preincubated in a shaking water bath for 30 min at 37 °C. At the end of the preincubation period the muscles were transferred to other incubation vials containing 1.5 ml of Krebs–Ringer bicarbonate buffer supplemented with 5 mM-D-[3-^{3}H]glucose (1 μCi/vial) (New England Nuclear, Dreichenheim, Germany) and insulin at the concentrations indicated above. Incubation was then continued for 60 min at 37 °C. In order to keep both the pH and the pO_2 constant throughout the preincubation and incubation periods the gas in the vials was saturated with O_2/CO_2 (19:1) and refreshed every 15 min; in between, the vials were closed air-tight. In a previous study it turned out that O_2 saturation was the same ($\geqslant 99.5\%$) whether gassing was intermittent or continuous.

Washing and digestion were carried out as described by Le Marchand-Brustel *et al.* [7]. Labelled glycogen was measured as described previously [5], and protein was determined by the method of Lowry *et al.* [13], with BSA as standard.

Muscle samples for histochemical studies were taken from both incubated [30 min ($n = 6$), 60 min ($n = 8$) and 90 min ($n = 6$)] and non-incubated [0 min ($n = 6$)] control muscles. They were mounted in an embedding medium (Tissue Tek; Miles Laboratories, Elkhart, IN, U.S.A.), frozen in isopentane cooled with liquid N_2 to its freezing point, and stored at −80 °C until analysis. Transverse sections (10 μm) were taken from the middle 'belly' region of the muscles with a cryostat at −22 °C and made to react with haematoxylin/eosin for a general view and periodic acid/Schiff reagent for glycogen. Next, the average maximal thickness was determined at the middle 'belly' region of the muscles. Because of the small absolute amount of glycogen in the muscles, we had to prepare a fresh Schiff reagent solution every day. The pH was measured every 15 min by a standard pH-meter

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Table 1. Parameters of incubation medium that are indicative of a stable muscle preparation *in vitro*

The values are expressed as means ± S.D., with the numbers of observations (*n*) in parentheses. The values for pH, osmolality and O_2 saturation of medium for soleus and extensor digitorum longus muscles are combined. The first 30 min coincided with the preincubation period, whereas the period from 30 min to 90 min coincided with the incubation period (see the Materials and methods section). The pH was checked a further 30 min after removal of the muscle from the incubation medium. Abbreviations: EDL, extensor digitorum longus; N.D., not determined.

Time (min)	Creatine kinase release (% of total muscle content)		pH	Osmolality (mosM)	O_2 saturation (% O_2 in medium)
	Soleus	EDL			
0	0.20±0.9 (12)	0.12±0.2 (12)	7.37±0.02 (40)	288±3.2 (12)	99.7±0.05 (12)
15	4.19±1.2 (12)	2.39±1.8 (12)	7.38±0.02 (37)	290±4.0 (11)	99.7±0.08 (12)
30	5.20±2.2 (12)	2.89±1.4 (12)	7.37±0.03 (40)	289±2.2 (12)	99.6±0.11 (12)
45	6.78±1.6 (12)	3.68±1.9 (12)	7.40±0.10 (40)	300±5.8 (8)	N.D.
60	8.96±3.4 (12)	4.93±2.8 (12)	7.40±0.09 (40)	290±5.1 (12)	99.4±0.05 (20)
90	8.32±2.8 (12)	5.03±1.8 (12)	7.41±0.11 (40)	287±1.3 (12)	99.5±0.06 (20)
120	N.D.	N.D.	7.40±0.10 (20)	N.D.	N.D.

(PHM 82 standard; Radiometer, Copenhagen, Denmark). Osmolality was measured by the method of freezing-point depression. In a separate study creatine kinase activity was measured in samples withdrawn from the incubation medium every 15 min and in the muscle homogenates at the end of the incubation period by using a Boehringer Mannheim G.m.b.H. (Mannheim, Germany) standard method. Values were corrected for volume changes of the medium.

RESULTS

Mean muscle weights (±S.D.) for soleus and extensor digitorum longus muscles were 7.6±1.3 mg and 9.4±2.2 mg respectively. The average largest diameter (±S.D.) for soleus muscle was 1.15±0.45 mm and that for the extensor digitorum longus muscle was 1.35±0.82 mm.

During incubation of the muscles under out experimental conditions, three important extracellular physiological parameters, namely pH, O_2 saturation and osmolality, appeared to be stable (Table 1). The release of intracellular enzymes, indicative of a situation of metabolic stress caused by physical or metabolic damage [6,14], was also low. The small fraction of creatine kinase totally released during the incubation period (< 10% of total muscle content for both soleus and extensor digitorum longus muscles) indicates that no severe stress was caused by the experimental procedures (Table 1).

Incorporation of D-[3-^{3}H]glucose into glycogen was linear at the lower insulin concentrations (0.4 and 1.0 munit/ml) but levelled off at a concentration considered to be supraphysiological (10 munits/ml). The effect of insulin on glycogenesis by the soleus and extensor digitorum longus muscles is shown in Fig. 1. Basal rate of glycogenesis (at 0 munit of insulin/ml) was approximately 2-fold greater in the soleus than in the extensor digitorum longus muscle. In the presence of 0.4, 1.0 and 10.0 munits of insulin/ml glycogenesis increased significantly in both muscles but remained 2–3-fold greater in the soleus than in the extensor digitorum longus muscle. At the lower (0.4 and 1.0 munit/ml) insulin concentrations there was a steep and progressive increase, then thereafter the curve levelled off, probably because of maximum insulin-receptor occupation.

Fig. 2(*b*), stained with periodic acid/Schiff reagent, shows that the incubated soleus muscle (30 min preincubation and 60 min incubation) demonstrated a central loss of glycogen, in contrast with the non-incubated control muscle (Fig. 2*a*). The same was found for the extensor digitorum longus muscle (result not shown). It was further observed that the appearance of the core

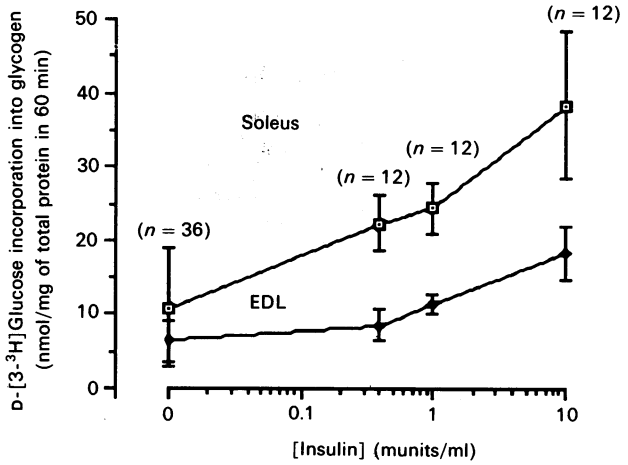

Fig. 1. Effects of insulin on soleus and extensor digitorum longus (EDL) muscles

Data are expressed as nmol of D-[3-^{3}H]glucose incorporation into glycogen (means ± S.E.M.). Muscles were incubated at 37 °C for 1 h preceded by a 30 min preincubation period. One muscle of each animal was used as test muscle [0.4, 1.0 and 10.0 munits of insulin/ml (*n* = 12)] and the contralateral muscle was used as control [0 munit of insulin/ml (*n* = 36)].

had already occurred after 30 min of preincubation (result not shown), indicating that core formation had already taken place during the preincubation period. Furthermore, the cells in the core lost their normal shape and positioning with respect to each other. In contrast with the control (Fig. 2*d*) they had a rounded shape, and were swollen and darker (Fig. 2*c*).

DISCUSSION

The aim of the present study was to evaluate a previously described intact skeletal-muscle model *in vitro* [4,5,7,9,11]. The incubated mouse skeletal muscles used in these studies were assumed to provide a suitable model in order to study various endocrinological effects on metabolism. Mouse soleus and extensor digitorum longus muscles, containing predominantly (75%) type 1 slow-twitch fibres (soleus) and predominantly (99%) type 2 fast-twitch fibres (extensor digitorum longus) [15], have been considered thin enough to allow an adequate delivery of O_2 and substrates *in vitro*. However, this assumption is merely

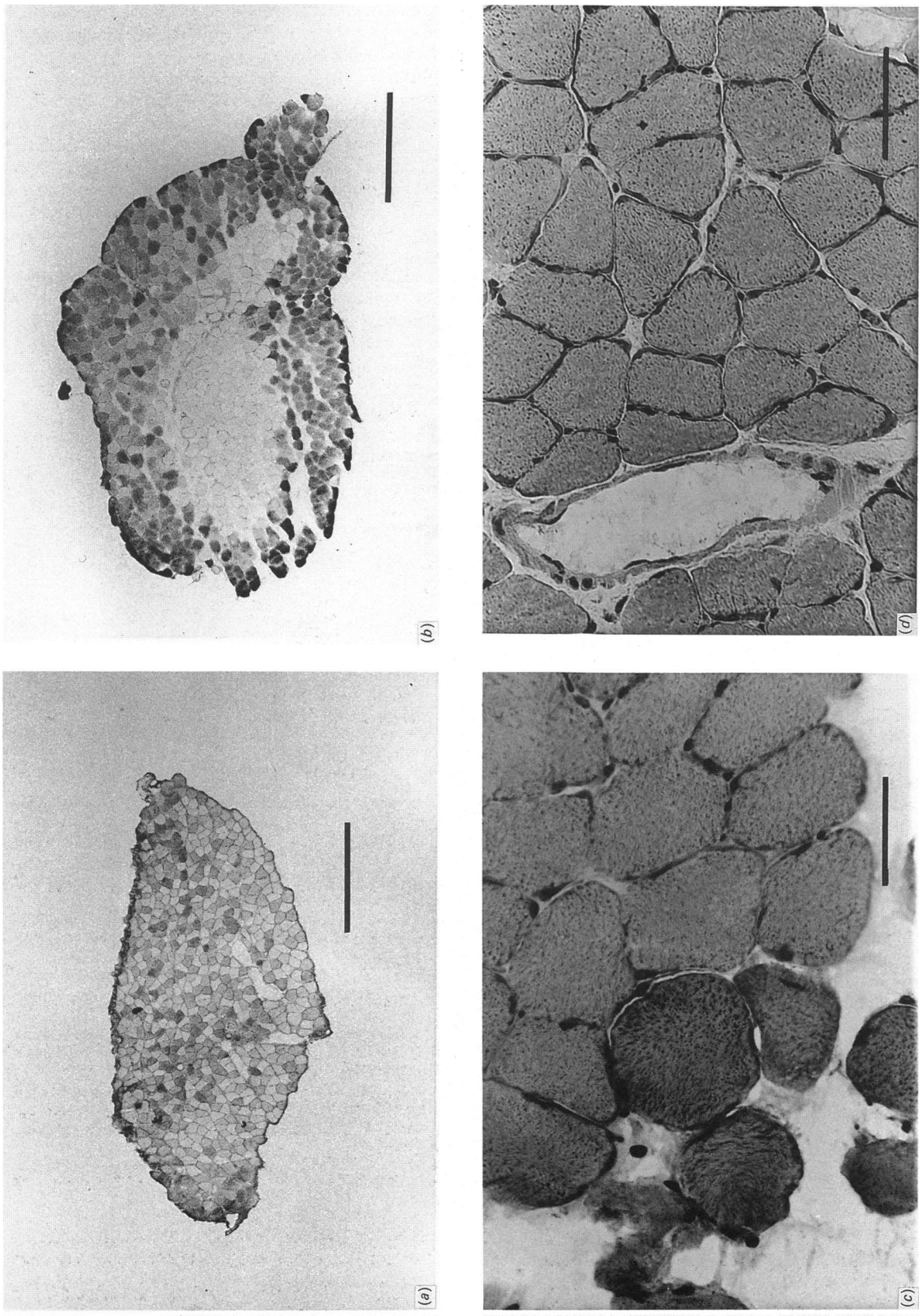

Fig. 2. Transverse sections through unincubated control soleus (*a*) (bar represents 0.35 mm) and soleus muscle incubated for 90 min (30 min preincubation and 60 min incubation) at 37 °C (*b*) (bar represents 0.30 mm), stained for glycogen (periodic acid/Schiff reagent)

The central core (*c*) (bar represents 0.03 mm) demonstrated darkened and swollen cells, in contrast with the control (*d*) (bar represents 0.05 mm), probably caused by hypoxia.

based on extrapolations of biochemical data on whole rat muscle or muscle-strip homogenates [16,17].

In the present investigation the data on D-[3-^{3}H]glucose incorporation into muscle glycogen agreed well with those reported by other investigators using the same model [4,5,7,11], with the exception that [^{3}H]glucose incorporation into glycogen was slightly less pronounced. This is probably due to the different feeding states (fed versus fasted) of the animals [7,18]. At all insulin concentrations studied insulin-stimulated glycogenesis was found to be greater in the soleus than in the extensor digitorum longus (Fig. 1), which is in line with previous investigations [4,5,11]. This difference in glucose uptake between both types of muscles is probably initiated by the different availability of glucose-transporter molecules, located at the muscle membrane [19]. However, the results of the present study showed a wide variance, which seems to be in keeping with other investigators [4,5,7].

A check on parameters of the incubation medium indicated that O_2 saturation, osmolality, pH and creatine kinase efflux from the muscles could not account for the observed inter-experimental variation. However, staining the muscles with haematoxylin/eosin and periodic acid/Schiff reagent showed the formation of large cores in both muscles, with swollen, darkened and isolated cells (Fig. 2). This core formation was time-dependent and was already observed after 30 min of pre-incubation. The core area differs markedly from one muscle to another, resulting in a wide variation of glucose incorporation. At this moment we have no explanation for this observation, but it is probably the individual physiological status of the animals that reflects this situation. Despite the fact that the soleus muscle is attached to the gastrocnemius muscle, and that a considerable amount of force is required to dissect the muscle free, the observed small creatine kinase efflux indicates that the difference in variation between the soleus and extensor digitorum longus muscles is not caused by the surgical technique. Core formation is probably caused by intramuscular hyperosmolality due to a hypoxic situation. As a consequence of the O_2 deficiency glycogen has been metabolized anaerobically in order to supply energy for intracellular processes, and consequently glycogenesis is impaired. Core formation was also observed by Maltin & Harris [16] in muscles of slightly larger animals (rats of body wt. 40–70 g). Our results support the findings by the latter investigators for even smaller animals (with higher metabolic rates), but contrast sharply with the observations and calculations made by Segal & Faulkner [12] and Newsholme *et al.* [17]. They suggested a diameter of 1.2 mm or less for rat muscle to be sufficient for oxygenation during incubation *in vitro*. The results of the present investigation show that this cannot be maintained for mouse muscles either, as already suggested by Maltin & Harris [16]. Hence either muscle strips or isolated muscle fibres should be used. However, strips of mouse muscle are hard to obtain, because of the small size of the muscle. Although isolated individual muscle fibres treated with collagenase as described by Zuurveld *et al.* [20] could be maintained and used under controlled conditions for the study of muscle metabolism under a wide variety of experimental interventions, this system has its drawbacks too. A major disadvantage of such a test system is the loss of the integrity of the tissue. Furthermore, it is not possible to evaluate the muscle viability by applying biochemical and histochemical techniques, such as in our study. Finally, it may be questioned whether the incubation (5.5 h) itself and/or treatment with collagenase will damage the muscle-fibre membrane, causing degradation of the insulin receptors.

In conclusion, the present study shows that the isolated mouse skeletal-muscle preparation is only of limited value for investigations of the effects of hormones (especially hormones with a less potent effect than insulin) on muscle metabolism, which is in line with the observations made by Maltin & Harris [3] for rat muscles. The viability of the muscle is not sufficiently preserved despite the stability of important biochemical parameters. Therefore viability of the muscle should be confirmed not only by biochemical assays but also by histochemical techniques to rule out any regional heterogeneity. Core formation seriously underestimates the rate of glycogenesis, which, in turn, may lead to misinterpretations of other biochemical and physiological events. Results of studies carried out *in vitro* with intact muscles from mice or larger rodents should thus be interpreted with caution.

The technical assistance of G. van Kranenburg and E. Terwindt, the enthusiastic and helpful comments of Dr. H. Kuipers and the support of A. Lammers are kindly appreciated.

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Received 1 November 1989/8 January 1990; accepted 31 January 1990