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A histochemical and morphological study of skeletal muscle from obese hyperglycaemic *ob/ob* mice

R. E. Almond and M. Enser

Muscle Biology Division, Agricultural Research Council, Meat Research Institute, Langford, Bristol, UK

Summary. The histochemical and morphological characteristics of muscles from lean and obese hyperglycaemic *ob/ob* mice were compared to determine the nature of the low skeletal muscle mass of the latter. Gastrocnemius and biceps brachii muscles from obese *ob/ob* mice were significantly lighter than those from lean mice, whereas the weights of soleus muscles were not significantly different. The small mass of the biceps brachii muscle resulted from a decrease in diameter of the large glycolytic fast white and fast intermediate fibres and a reduction in the proportion of fast white fibres. The size and frequency of the more oxidative fast red fibres was not different. The histochemical appearance of all fibre types studied from muscles in obese mice was identical to equivalent fibres in lean mice. The fat content of muscles from obese mice was three times greater than in lean mice whereas muscle DNA concentration was similar.

Key words: Obese mice, muscle size, fibre type, fibre number, diabetes.

Obese hyperglycaemic mice with the *ob/ob* genome have been used extensively as a model for Type 2 (non-insulin-dependent) diabetes. They are characterized by many abnormalities including hyperphagia, fasting hyperglycaemia, and hyperinsulinaemia. They have fewer insulin receptors and their tissues, particularly muscle, show a marked resistance to the normal metabolic actions of this hormone [1]. Obese mice have similar quantities of total body protein to lean mice [2]. However, extra protein present in their enlarged adipose tissue, liver, and intestine disguises a lower content of skeletal muscle protein [3, 4].

The smaller amount of muscle could be due to a decrease in the number of muscle fibres, or a reduced volume per fibre, or both. If there were fewer fibres within the muscles of obese mice, it would indicate a difference in pre-natal development compared with lean mice since in mice the majority of muscle fibres are present at or soon after birth [5, 6]. On the other hand, a decrease in the post-natal growth of fibres would be compatible with the other reported metabolic defects in *ob/ob* mice. Insulin is probably the most important physiological factor regulating the overall protein balance in skeletal muscle [7] since it not only stimulates the uptake of amino-acids and protein synthesis, [8] but also inhibits protein degradation. It is conceivable, therefore, that the inherent resistance of muscles from obese mice to the anabolic effects of insulin [9, 10] could facilitate a decrease in the post-natal growth of this tissue. Plasma corticosteroid levels are also elevated in *ob/ob* mice [11, 12] and may cause accelerated protein degradation and diversion of amino-acids into excessive gluconeogenesis [13]. If these metabolic abnormalities in obese mice are responsible for their decreased skeletal muscle mass, one would predict that fast and slow phasic muscles would respond differently, since either streptozotocin-induced diabetes [14] or corticosteroid injections in rats [15] cause large glycolytic fibres and fast phasic muscles, to lose most weight.

We investigated the number, type, and size of fibres in the biceps brachii muscles of lean and obese mice to determine whether the smaller muscles in the latter show differential changes in fibre size and type. The biceps brachii muscle was chosen since it contains a good mixture of red and white fibres. We also report more limited studies on the gastrocnemius, a muscle containing predominantly fast white fibres, and the soleus, a slow phasic red muscle which contains approximately equal numbers of slow and fast red fibres. The weight of muscles in mice from 3 to 48 weeks of age was studied to determine whether the small muscles [3, 4] in young obese animals show 'catch-up' growth as the degree of diabetes changes [16].

Materials and methods

Animals

Obese mice of the C57 Bl/6J strain, homozygous for the *ob* gene, were bred in our colony from stock obtained from the Jackson Laboratory, Bar Harbor, Maine, USA. Lean controls (+/+) were obtained from a sub-line lacking the *ob* gene. Animals were maintained in a temperature-controlled environment and fed a normal pelleted diet (Oxoid, London, UK) and water ad libitum. Animals were killed by cervical dislocation while under ether anaesthesia or by exposure to chloroform vapour.

Table 1. Histochemical classification of muscle fibre types

	Relative enzyme activities		
Muscle fibre type	Alkali-stable ATPase	Succinate dehydrogenase	Phosphorylase
Fast white	+	+	+ + +
Fast intermediate	+, or + +	+ +	+ +, or + + +
Fast red	+ + +	+ + +	0, or +
Slow red	0	+ +	0

0→+ + + increasing levels of relative enzyme activities as judged subjectively

Histological procedures

Muscles were rapidly excised, weighed, and frozen by immersion in isopentane cooled to −160 °C in liquid nitrogen [17]. After equilibration at −20 °C, transverse sections (10 μm thick) were cut from the middle of the muscle and air-dried for up to 30 min. Sections were either used immediately or stored overnight in an air-tight container at −20 °C. Serial sections were then stained for alkali-stable ATPase [18], succinate dehydrogenase [19], or phosphorylase [20]. Fibre number determinations were carried out on sections stained with dilute Grams iodine [21] which served to outline the fibres.

Microscopy and photography

Microscopic observations were made using a Laborlux 12 microscope (Leitz, Wetzlar, FRG), fitted with a Leitz ×16 bright field planachromatic objective lens. Photographs were recorded on Ilford pan F film (50 ASA). Magnifications were determined from photomicrographs taken off a calibration graticule.

Table 2. Weight of muscles from lean and obese male mice

				Muscle					
Age (weeks)	Mouse type	Weight of mice (g)	*p*	Biceps brachii (mg)	*p*	Gastrocnemius[a] (mg)	*p*	Soleus	*p*
3	Lean	9.7±0.9 (5)		4.4±0.4 (4)		–		–	
	Obese	11.1±0.5 (5)	NS	4.0±0.1 (4)	NS	–	–	–	–
8	Lean	23.9±0.4 (15)		12.9±0.2 (30)		133.1±4.0 (14)		9.9±0.8 (5)	
	Obese	35.4±0.6 (15)	<0.001	9.3±0.2 (30)	<0.001	109.6±3.1 (14)	<0.001	9.9±0.4 (5)	NS
12	Lean	27.4±0.5 (6)		13.3±0.4 (12)		152.8±1.7 (12)		–	
	Obese	47.6±2.0 (6)	<0.001	11.5±0.3 (12)	<0.01	118.5±3.4 (12)	<0.001	–	–
24	Lean	31.4±0.9 (5)		15.7±0.3 (10)		174.2±4.5 (10)		–	
	Obese	58.6±1.6 (5)	<0.001	13.9±0.3 (10)	<0.001	130.9±3.9 (9)	<0.001	–	–
48	Lean	33.6±0.8 (5)		16.1±0.2 (10)		167.9±1.3 (10)		–	
	Obese	71.1±1.1 (6)	<0.001	13.4±0.3 (12)	<0.001	138.4±1.4 (11)	<0.001)	–	–

Results expressed as mean±SEM with the number of animals in parentheses.
[a] The flexor digitorium superficialis is included with the gastrocnemius

Table 3. Fibre number, DNA and fat content of muscles from 8-week-old-male mice

	Obese	Lean	*p*
Mouse weight (g)	34.8 ± 1.1 (9)	21.1 ± 0.7 (8)	<0.001
Blood glucose (mmol/l)	12.8 ± 0.4 (9)	10.4 ± 0.2 (8)	<0.001
Biceps Brachii			
DNA (μg/muscle)	8.6 (33)	12.0 (30)	
Muscle/DNA (mg/μg)	1.10 (33)	1.06 (30)	
Fat % by weight	4.2 (6)	1.4 (6)	
Fibres per muscle	2794 ±343 (7)	2848 ±232 (7)	NS
Gastrocnemius			
DNA (μg/muscle)	37.8 ± 2.3 (7)	47.2 ± 3.5 (7)	<0.01
Muscle/DNA (mg/μg)	2.8 ± 0.1 (7)	3.0 ± 0.2 (7)	NS
Fat % by weight	4.04 ± 0.3 (7)	1.40 ± 0.1 (7)	<0.001
Soleus			
Fibres per muscle	883 ± 43 (6)	795 ± 57 (6)	NS

Results are expressed as mean ± SEM with the number of animals in parentheses. Where SEM is absent results are the mean of a triplicate determination on pooled muscles

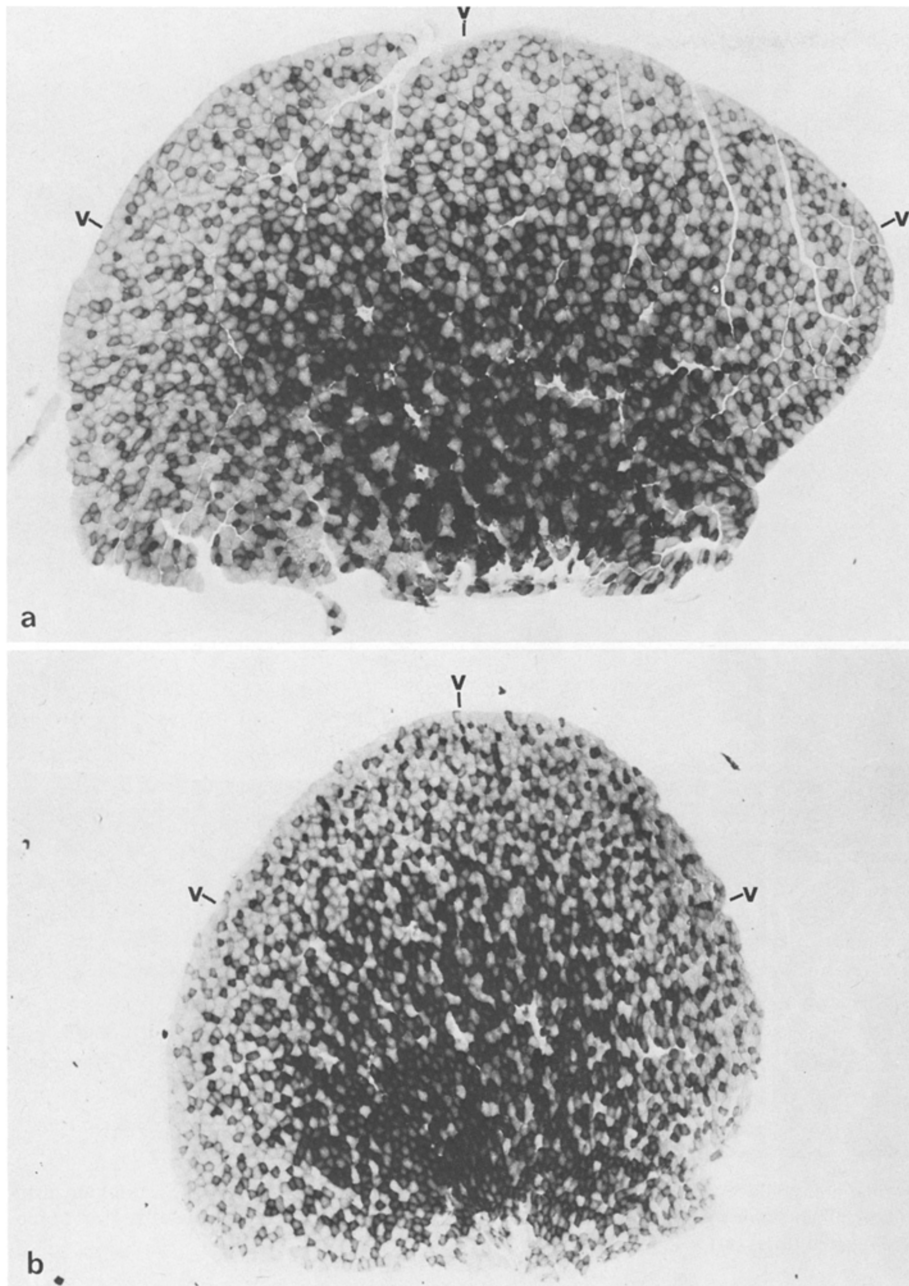

Fig. 1 a and b. Complete transverse sections of biceps brachii muscles from **a** lean and **b** obese male mice, stained for succinate dehydrogenase. Note predominance of fast white fibres around the ventral periphery (V) of both muscles; and the relative areas of each muscle occupied by these lightly staining fibres (× 40)

Fibre sampling and measurement

To overcome the asymmetric distribution of fibres within the biceps brachii muscle, we used a sampling system based on two grids composed of equidistant parallel lines spaced 2.5 cm apart. The grids were superimposed at right angles to one another randomly upon photomicrographs (overall magnification × 270) of complete muscle transverse sections. A cluster of four to six fibres was then counted at each intercept of the two sets of grid lines. Muscle fibre diameters were measured with micrometer calipers, directly from enlarged photomicrographs of sections (× 270) stained for alkali-stable myosin ATPase, by taking the mean of two measurements made at orthogonal angles.

Fibre type classification and nomenclature

Fibres were identified in serial, histochemically stained, sections and the degree of staining recorded for each enzyme activity independently. The fibres were then classified as fast white, fast intermediate, fast red or slow red, according to the criteria shown in Table 1. The nomenclature used to describe these four muscle fibre types is a slight modification of that originally used by Gauthier and Lowey [22]: this nomenclature has the advantage that it characterises a fast intermediate fibre type.

Estimation of DNA and fat content of muscles

Muscles were dissected out rapidly after death and weighed. They were analysed immediately or stored at −20 °C. Fat content was determined as fatty acids [23] after saponification of the whole muscle, assuming a mean molecular weight of 281. DNA was determined by the method of Burton [24] with calf thymus DNA as standard.

Blood glucose determination

Samples of blood (20 μl) were obtained from the retro-orbital plexus of fed mice under ether anaesthesia between 11.00 and 13.00 h. After deproteinization by the barium hydroxide/zinc sulphate procedure,

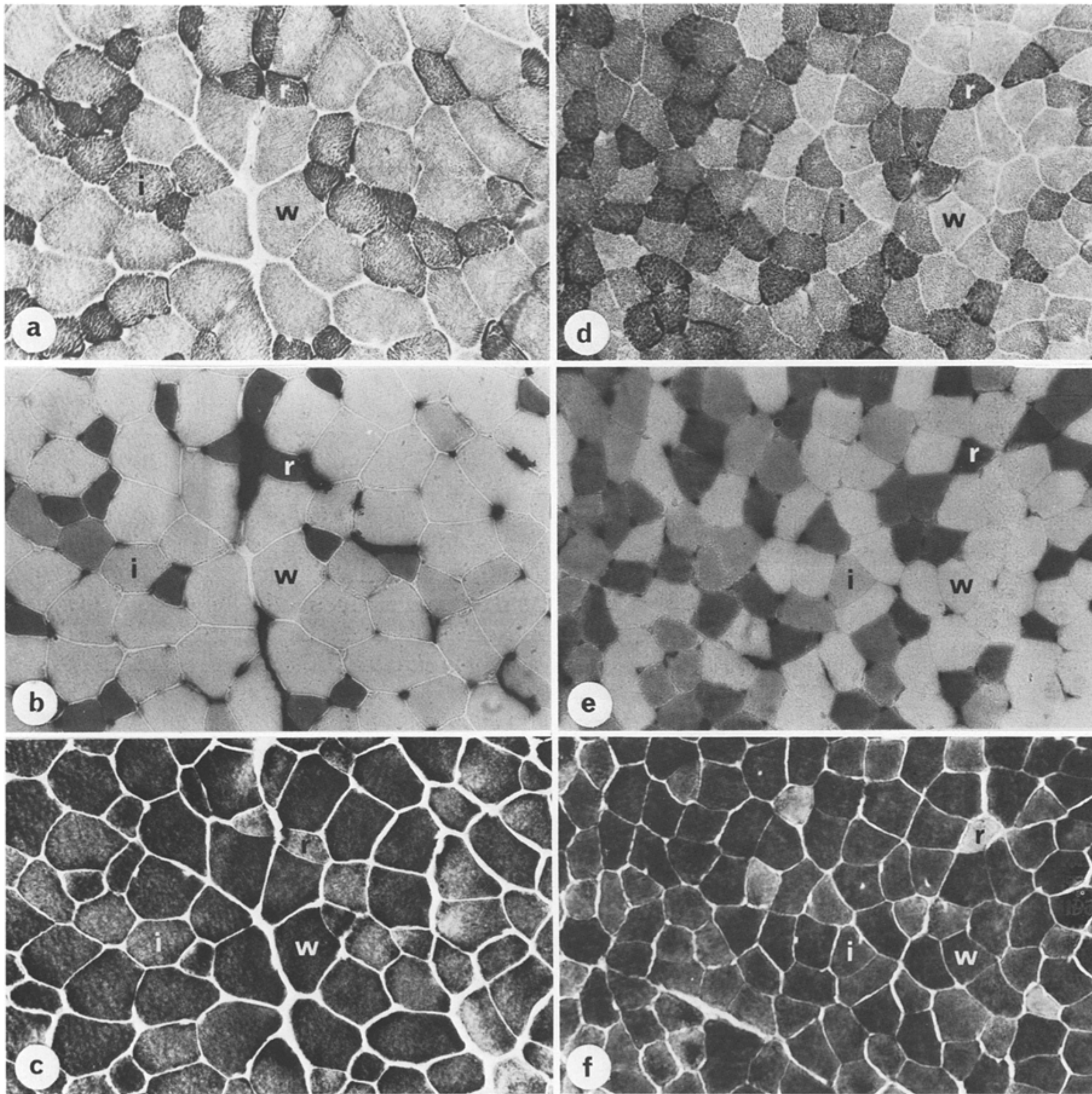

Fig. 2 a–f. Serial transverse sections taken from similar areas of biceps brachii muscles of lean **(a–c)** and obese male mice **(d–f)**. Sections are histochemically stained for succinate dehydrogenase **(a, d)**, alkali-stable myosin ATPase **(b, e)**, and phosphorylase **(c, f)**. Note the relative sizes of representative fast white (w), fast intermediate (i), and fast red fibres (r) (× 225)

glucose was measured by the glucose oxidase technique using 2.2′-azino-di-(3-ethyl-benzthiazoline sulphonate (6) (Boehringer, Lewes, Sussex, UK) as the chromophore.

Statistical analysis

The mean ± SEM of the data were evaluated and compared using the unpaired two-tailed Student's t-test.

Results

Muscle weight and composition between 3 and 48 weeks of age

At 8 weeks of age the biceps brachii and gastrocnemius muscles from obese mice were significantly lighter than those from lean mice, whilst the soleus muscles from the same animals showed no significant difference in weight (Table 2). The weight differences between both biceps brachii and gastrocnemius muscles from lean and obese animals were maintained up to at least 48 weeks of age, and there was no consistent evidence of 'catch-up' growth during this period by muscles from obese animals.

The quantity of DNA in the muscles from obese mice was less than in the corresponding muscles from lean mice (Table 3). However, when calculated as muscle mass per unit of DNA, the results became virtually identical. In all cases biceps brachii muscles contained a greater concentration of DNA than gastrocnemius muscles.

The fat content of the gastrocnemius and biceps brachii muscles from obese mice was approximately

Table 4. Numbers and size of muscle fibre types in the biceps brachii from lean and obese male mice

	Type of mouse	Type of fibre		
		Fast white	Fast red	Fast intermediate
Percentage distribution in 3-week-old-mice	Lean ($n=6$)	31.0±1.5	30.0±2.3	38.2±1.5
	Obese ($n=6$)	26.3±1.4	31.6±2.4	41.3±2.9
	p	<0.05	NS	NS
Percentage distribution in 8-week-old-mice[a]	Lean ($n=6$)	43.9±0.9	32.6±1.0	23.1±0.9
	Obese ($n=6$)	38.9±0.9	34.0±1.4	26.6±1.2
	p	<0.001	NS	<0.05
Fibre diameter in 8-week-old-mice[b] (μm)	Lean ($n=6$)	50.8±2.9	34.2±1.0	42.4±0.9
	Obese ($n=6$)	43.0±1.0	31.4±1.0	38.2±0.9
	p	<0.001	NS	<0.01

Results are expressed as mean ± SEM with the number of animals in parentheses. [a] Approximately 600 fibres typed per muscle. [b] Diameters determined on at least 20 fibres of each type in six muscles from each type of mouse

three times greater than that in muscles from lean mice and slightly obscures the true extent of muscle protein loss in these animals when comparing figures based on muscle wet weight (Table 3).

Number, size, and type of muscle fibres

The total number of fibres in either the biceps brachii or soleus muscles was not significantly different between lean and obese mice (Table 3). The characteristic asymmetric arrangement of fibre types within the biceps brachii muscle, with a clustering of fast red fibres in the central dorsal area, was also similar for lean and obese mice (Fig. 1). No qualitative differences in histochemical staining response for succinate dehydrogenase, alkali-stable ATPase or phosphorylase were observed in fibres of the same type from lean or obese animals (Fig. 2). However there was a difference, in the case of the biceps brachii muscle, in the relative proportions of their constituent fibre types (Table 4). In 8-week-old-lean mice, fast white fibres were present in the greatest numbers (44%), followed in turn by fast red (33%), and fast intermediate (23%) fibres. In 3-week-old-mice, the frequency distribution of fibre types was somewhat different; there being a greater proportion of fast intermediate fibres (38%) and a decreased frequency of fast white fibres (31%), whilst the proportion of fast red fibres (30%) was similar to that observed in the older mice. Biceps brachii muscles from obese mice showed very similar developmental changes in fibre composition between 3 and 8 weeks of age (Table 4), but at both ages the obese animals contained significantly fewer fast white fibres and more fast intermediate fibres, whilst the number of fast red fibres was not significantly different from that observed for lean mice. In the biceps brachii muscle from lean mice fast white fibres had a mean diameter of 50.8 μm, fast intermediate fibres a mean diameter of 42.4 μm and fast red fibres were smallest with a mean diameter of 34.2 μm. The compositional differences observed in the muscles from obese animals, when considered in relation to the sizes of the different fibre types, are insufficient to explain fully the lower muscle weight in these animals. Since the total number of fibres was found to be similar in both sets of mice, the discrepancy in muscle weight must be due to changes in fibre diameter. Observations of transverse sections of biceps brachii muscles from both types of mice indicated that the major differences in fibre diameter were occurring around the ventral periphery of the muscle, a region which contains predominantly fast white fibres (Fig. 1). Measurements of fibre diameters from these muscles confirmed that whilst all fibre types from obese animals showed reduced diameters, in comparison with similar fibres from lean mice (Fig. 2), this difference was significant only in the larger more glycolytic fast white and fast intermediate fibres whilst the smaller fast red fibres, which have a greater reliance on oxidative metabolism, were not significantly reduced in diameter with respect to similar fibres from lean mice (Table 4).

Discussion

Previous studies, which have reported decreased skeletal muscle mass in obese mice [3, 4], have not examined individual muscles. Whereas our results clearly show a significant reduction in weight of both the biceps brachii and gastrocnemius muscles of obese mice, the soleus muscles from the same mice were of identical weight to those from lean mice. A more detailed study of the biceps brachii muscle suggested that the lower muscle protein content in obese mice was not achieved in a uniform way but differed between fibres of different types. The bulk of the reduced skeletal muscle mass was accounted for by a reduction in size of the larger more glycolytic, fast white and fast intermediate fibres and to a lesser extent by a decrease in the frequency of fast white fibres. The weight differences we have reported in the biceps brachii muscles from 8-week-old-lean and obese mice are consistent with the differences in the size and distribution of fibre types. The muscles from lean mice used for fibre size determination and distributions were 32% heavier than the obese muscles. How-

ever the obese muscles also contained 3% extra fat by weight and if this is taken into account the difference in lean muscle mass becomes 36% whilst the total muscle cross-sectional area calculated from the data was 37% greater for lean mice.

That there are a similar number of fibres in the biceps brachii muscle from lean and obese mice indicates that the low muscle weight of obese mice results from a post-natal metabolic abnormality. The lower proportion of large fast white fibres and the concomitant increase in the number of fast intermediate fibres within the biceps brachii of obese mice, initially appears to suggest a retarded development of some of the fast intermediate fibres into fast white fibres. However the change in distribution of fibre types which occurred between 3 and 8 weeks of age was very similar in both obese and lean mice and maintained a difference that was already established at 3 weeks of age. The subsequent lack of 'catch-up' growth by the biceps brachii of obese mice does not lend support to the hypothesis of delayed maturation but indicates a persistent metabolic defect.

The differential growth between the smaller biceps brachii and gastrocnemius and the normal sized soleus muscles, in obese hyperinsulinaemic mice, is similar to the differential effects observed by Armstrong et al. in insulin-deficient streptozotocin-diabetic rats [14, 25], where a reduction in size of the fast phasic gastrocnemius muscle was mediated by a decrease in size of the larger more glycolytic fibres, whilst the slow oxidative fibres were not significantly affected. The activity of an alkaline protease, which is reported to be increased in the muscles of diabetic rats [26], is also elevated in the muscles of obese compared with lean mice [27]. The significance of this enzyme in the turnover of myofibrillar proteins, however, has been questioned [28].

Perhaps of relevance in this context, circulating glucocorticoids are reported to be elevated in both diabetic rats [29] and obese mice [11, 12] before 3 weeks of age, and have been shown to cause decreased synthesis of DNA and the preferential loss of white muscle [15, 29]. Further evidence for the involvement of corticosteroids has been provided by work using adrenalectomized mice. Shimomura et al. [30] have reported that adrenalectomy arrests the further development of obesity whilst gastrocnemius muscles from these adrenalectomized *ob/ob* mice were significantly heavier than those from intact obese animals.

It is possible that hormones other than insulin or glucocorticoids contribute to the decreased skeletal muscle mass in obese mice. Thyroxine levels are depressed in 6–20-day-old *ob/ob* mice [31], although they are normal after weaning [32]. Circulating growth hormone is reported to be normal [33] or decreased [34] in obese mice, but may not contribute to the decrease in skeletal musculature since plasma somatomedin concentration is normal [35]. It could be argued also that the marked hypoactivity of obese mice may trigger a reduction in size of their presumably less frequently used fast twitch skeletal muscle fibres. However, the soleus muscle is a continually active postural muscle, and constant contractile activity has been shown to be a fundamental determinant of muscle mass which can take precedence over endocrine signals for muscle protein depletion [15, 36]. Decreased long bone growth in obese mice is unlikely to be the cause of the decreased muscle size since the soleus and gastrocnemius muscles share the same tendon but only the latter is smaller.

We conclude that the reduced skeletal musculature in obese mice results from a decrease in muscle fibre diameter which occurs in a specific manner and affects large glycolytic fibres most and small oxidative fibres least. It remains to be determined whether insulin resistance or the elevated corticosteroid levels in these mice are responsible for their reduced skeletal muscle mass.

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Dr. M. B. Enser
AFRC Meat Research Institute
Langford
Bristol BS18 7DY
UK