

The effect of acute dietary restriction on muscle fibre number in weanling rats*

BY DONALD K. LAYMAN†, PATRICIA B. SWAN
AND P. V. J. HEGARTY

*Department of Food Science and Nutrition, University of Minnesota, St Paul,
Minnesota 55108, USA*

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1. Male Sprague-Dawley rats were allocated at 100 g into either an *ad lib.*-fed control group or a food-restricted group. The restricted group was fed for 9 d at 25% of *ad lib.* intake. Controls were killed at a body-weight of 100 g and 29 d of age and the restricted animals were killed at 70 g and 38 d of age.
2. The effects of food restriction on muscle weight, fibre number, fibre diameter, DNA, and protein were examined in three skeletal muscles, the soleus, plantaris and extensor digitorum longus (EDL).
3. Acute dietary restriction caused body- and muscle-weight loss and a decrease in both the number and cross-sectional area of muscle fibres in each of the muscles.
4. The restriction halted growth-related increases in DNA in all muscles and decreased the protein:DNA value in the plantaris and EDL.
5. These results indicate that present theories describing cellular development are not adequate to define growth potential or growth retardation of skeletal muscle.

Permanent growth retardation resulting from temporary nutritional restriction is believed to be related to a decrease in cell number (Winick & Noble, 1966). These authors categorized tissue growth into three phases: (1) hyperplasia, increases in the number of cells, (2) hyperplasia-hypertrophy, concomitant increases in cell number and cell size, and (3) hypertrophy, growth in the size of existing cells with no change in the number of cells. Therefore, any nutritional insult which inhibits cell growth during the period of hyperplasia will result in growth retardation due to a permanent reduction in the number of cells. On the other hand, dietary restrictions during hypertrophic growth affect cell size only, and the effects are usually reversed upon refeeding. This proposed pattern of tissue development has been challenged recently (Sands *et al.* 1979).

Quantitative measurements of cellular growth are usually made using nuclear number (DNA) as an estimate of hyperplasia and the protein:DNA value as an estimate of hypertrophy. These factors have been utilized to describe growth of mononucleated cells as found in brain, kidney (Winick & Noble, 1966), and adipose tissue (Knittle & Hirsh, 1968). However, for the multinucleated muscle fibre, the relationships of nuclear number and protein:DNA to growth potential and growth retardation is unknown. Generally, growth potential of skeletal muscle is estimated by counting the number of muscle fibres, and fibre number is regarded as reaching a constant value at, or shortly after, birth (Enesco & Puddy, 1964; Rowe & Goldspink, 1969). However, evidence that fibre number is not constant after birth has been reported for the rat (Rayne & Crawford, 1975; Layman *et al.* 1980), guinea-pig (Maxwell *et al.* 1973), chickens (Montgomery *et al.* 1964), dog (Ihemelandu, 1980), and human (Montgomery, 1962). Therefore, the significance of fibre number to growth potential remains controversial and the relationship of nuclear number and protein:DNA to fibre number and fibre size, respectively, is uncertain.

Nutritional restriction during early postnatal growth leads to growth retardation of

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† Present address: Department of Foods and Nutrition, University of Illinois, Urbana, Illinois 61801, USA.

Table 1. *Composition of the diet g/kg diet*

Component	g/kg of diet
Casein	280
D-glucose	258
Maize starch	258
Maize oil	100
Salt mixture*	50
Vitamin mixture†	44
α -Cellulose	10

* Salt mixture (Rogers-Harper g/kg): ammonium molybdate. 4 H₂O 0.03, calcium carbonate 292.9, calcium phosphate. 2 H₂O 4.3, cupric sulphate 1.56, ferric citrate. 6 H₂O 6.23, magnesium sulphate. 7 H₂O 99.8, magnesium sulphate. H₂O 1.21, potassium iodide 0.005, potassium phosphate 343.1, sodium chloride 250.6, sodium selenite. 5 H₂O 0.02, zinc chloride 0.2.

† Vitamin diet fortification mixture 23431 (g/kg): α -tocopherol 5.0, ascorbic acid 45.0, choline chloride 75, D-calcium pantothenate 3.0, *myo*-inositol 5.0, menaphthone 2.25, nicotinic acid 4.5, *p*-aminobenzoic acid 5.0, pyridoxine hydrochloride 1.0, riboflavin 1.0, thiamin hydrochloride 1.0. (mg/kg): retinyl acetate equivalent to 270 retinol, ergocalciferol 2.5, biotin 20, pteroylmonoglutamic acid 90, cyanocobalamin 1.35.

skeletal muscle (Winick & Noble, 1966; Widdowson, 1970). The effect of this restriction on cellular development remains in dispute (Sands *et al.* 1979). The present study examines the effects of acute dietary restriction on the number and cross-sectional diameter of muscle fibres. A 9 d partial restriction of food resulting in a 30% reduction of body-weight was utilized to retard cellular growth severely in skeletal muscles of young rats. A new technique which allows for rapid determination of muscle fibre number (Thompson *et al.* 1979) was utilized to compare actual counts of fibre number with the common biochemical estimates of cell (nuclear) number. This study is the first to examine the effect of nutritional restriction on fibre number and size as well as DNA and protein in the rodent.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Holtzman Co., Madison, WI, USA) weighting from 60 to 65 g were purchased at 21 d post partum. All animals were housed individually in stainless-steel, wire-bottom cages in a temperature- and humidity-controlled laboratory. Room lighting consisted of equal 12-hour periods of light and dark. Throughout the study all animals received water *ad lib*. All animals received the diet described in Table 1 *ad lib*. until they reached a body-weight of 100 g. Then animals were assigned to either control (C), partial-restriction (PR), or age control groups which had average body-weights of 100 \pm 2 g, 99 \pm 2 g and 97 \pm 3 g respectively. Each group consisted of fifteen animals. Control animals were killed at 29 d of age at body-weights of approximately 100 g. The PR group received 25% of the control *ad lib*. intake for 9 d in a single feeding at the beginning of each dark period. The PR group weighed approximately 70 g when killed. The age control group received the diet for 9 d and weighed 179 g at death. Based on preliminary experiments, it was estimated that virtually all animals would die when they had lost approximately 45% of body-weight which would have occurred within the following 48 h.

Postmortem procedures

Immediately after death, the heart, liver and three skeletal muscles from one side were removed from each animal. The skeletal muscles were: the soleus, plantaris, and extensor digitorum longus (EDL). These muscles were identified (Greene, 1959) and dissected from

Table 2. *Weight of skeletal muscles, heart and liver from control (C) rats, and from rats whose food intake was partially-restricted (PR)*

(Mean values and standard deviations for muscles from fifteen animals)

Tissue	Group	Tissue wet wt (mg)			Tissue wt:body-wt	
		Mean	SD	% Change	Mean	% Change
Soleus	C	41	2		41	
	PR	37	1	-8	53	+29
Plantaris	C	89	2		90	
	PR	68	4	-24	97	+8
Extensor digitorum longus	C	43	2		43	
	PR	32	2	-26	45	+5
Heart	C	497	6		500	
	PR	363	11	-27	516	+3
Liver	C	4700	200		4733	
	PR	2000	250	-57	2841	-40

origin to insertion. All adhering fat and connective tissue including tendons were removed from each of the muscles before weighing. These muscles were used for the measurements of protein and DNA concentrations. For the chemical assays the tendons were removed from each muscle and individual muscles (e.g. soleus) from one side of five animals were pooled to obtain a sufficient sample size. Pooled samples were stored in 5 vol. 0.25 M-potassium chloride for 3 d at -4° . Samples were then thawed on ice and an equal volume of cold trichloroacetic acid (200 g/l) was added. Samples were homogenized using a Brinkman PCU-2 Polytron homogenizer for 45 s and triplicate portions of the homogenate were assayed for DNA and protein according to the method of Schmidt & Thannhauser (1945), as modified by Munro & Fleck (1966), retaining the original lipid extraction. The DNA fraction was assayed by the indole reaction as described by Ceriotti (1952) and protein was determined by the method of Lowry *et al.* (1951).

Contralateral muscles were removed after the onset of rigor mortis and stored individually in formalin (100 ml/l) buffered with 0.1 M-phosphate buffer, pH 7.4, for at least 10 d before being used for determination of muscle fibre number and size. Fibre number and diameter were determined as described by Thompson *et al.* (1979). Evaluation of statistical significance of differences was performed using the Student's *t* test (Snedecor & Cochran, 1956).

RESULTS

Body-weight of rats in the control group averaged 99.3 ± 0.2 g and the rats were 29 d of age at the time of killing, whereas the rats in the PR groups given only 25% of the *ad lib.* intake for 9 d weighed 70.4 ± 0.1 g and were 38 d of age when killed.

Each of the muscles lost weight as the result of the dietary restriction (Table 2), the loss ranged from 8 to 26% of the weight at the beginning of the restriction. The soleus appeared to be somewhat spared the effects of food restriction, whereas the plantaris and EDL weight loss was more closely related to the extent of loss in body-weight. The heart lost 27% of its weight during the restriction while the liver lost 57% of its weight.

The results in Table 3 suggest that the loss in muscle mass due to acute dietary restriction results both from a decrease in the number of fibres in the muscle and a decrease in the size of the fibres which remain. The reduction in the number of fibres *v.* fibre diameter varied greatly among the muscles. The number of muscle fibres was reduced by 30% in the soleus

Table 3. *The number and diameter of muscle fibres from control (C) rats and from rats whose food intake was partially-restricted (PR)*
(Mean values with their standard errors)

Muscles	n	Group	Fibre no.			Fibre diameter (μm)		
			Mean	SE	% Change	Mean	SE	% Change
Soleus	5	C	3322	204		32.8	0.5	
		PR	2329	332*	-30	30.0	1.6	-8
Plantaris	8	C	6153	283		29.5	0.8	
		PR	5873	210	-5	25.0	0.7*	-15
Extensor digitorum longus	8	C	5315	257		23.5	0.3	
		PR	4216	246*	-21	20.5	0.4*	-13

* Mean values were statistically significant from central values: $P < 0.05$.

Table 4. *DNA and protein content of skeletal muscles from control (C) rats, and from rats whose diet was partially-restricted (PR)*
(Mean values and standard deviations)

Skeletal muscles	Group	DNA (μg): whole muscle			Protein (mg): whole muscle			Protein (mg):DNA (mg)		
		Mean	SD	% Change	Mean	SD	% Change	Mean	SD	% Change
Soleus	C	42	1		5.86	0.82		131	21	
	PR	43	2	+2	5.70	0.16	-3	134	14	+2
Plantaris	C	66	9		13.0	0.83		190	23	
	PR	69	2	+4	10.8	0.51	-17	162	13	-15
Extensor digitorum longus	C	33	3		6.63	0.95		191	45	
	PR	32	1	-3	5.31	0.35	-20	164	15	-14

during the dietary restriction, whereas the number of fibres was reduced by 21% in the EDL. Reduction in the numbers of fibres in the plantaris was less, however the reduction of fibre diameter was greater than in the two other muscles.

The DNA content of the soleus, plantaris, and EDL remained constant throughout both dietary restrictions (Table 4). During the period of the dietary restriction the DNA contents of the soleus, plantaris and EDL muscles increased to 53, 89 and 42 mg/muscle in the age controls. The percentage loss in protein (Table 4) corresponded well with the percentage loss in muscle weight (Table 2) with the soleus losing only 3% of its protein mass while the plantaris and EDL lost 17 and 20% respectively. Due to this loss of protein, the protein:DNA value decreased in the plantaris and EDL. Protein (μg tissue) remained constant during food restriction (values not shown).

DISCUSSION

The findings of this study indicate that during acute dietary restriction both the number of muscle fibres and the size of the fibres can be reduced. This is the first report of a postnatal decrease in the number of muscle fibres due to a nutritional stress. Fibre number decreased in the soleus, plantaris and EDL by 30, 5 and 21% respectively by the end of the 9 d

restriction. There was also a decrease in fibre size as measured by fibre diameter in each of the muscles. This decrease was significant in the plantaris and EDL.

Few studies have determined muscle fibre number after restriction of food intake and there is a lack of agreement among these studies. However, this lack of agreement is not surprising since these studies differ greatly in types of muscles, species and nutritional restrictions utilized (Montgomery *et al.* 1964; Rowe, 1968; Stickland *et al.* 1975; Kim & Hegarty, 1978). Previous reports indicate that nutritional restrictions, sufficiently severe to cause loss of body-weight, will cause a decrease in mean fibre diameter (Rowe, 1968; Kim & Hegarty, 1978). Goldspink (1965) suggested that a reduction of fibre diameter was due to a decrease in the number and size of myofibrils. Using an experimental design similar to the present one, Rowe (1968) examined the effects of partial starvation on five muscles of mature mice and concluded that during food restriction the number of muscle fibres remained constant but mean fibre diameter was reduced. On the other hand, a preliminary report by Kim & Hegarty (1978) indicated a decrease in both fibre number and mean fibre diameter in rats after prolonged total starvation. Similar controversy exists in studies which result in reduction of body-weight compared to age controls. Stickland *et al.* (1975) examined a single skeletal muscle from growing pigs after 1 year of food restriction. They reported that fibre number remained constant and that fibre diameter was lower than *ad lib.*-fed age controls. However, Montgomery (1962) reported a lower number of muscle fibres in infants who died from protein-energy malnutrition *v.* normal infants who had died in accidents.

The results in Table 3 suggest that the major reasons for lack of agreement among previous reports are: (1) large variations in the number of fibres in muscles from animals in the same treatment and (2) the failure of all muscles to respond identically to a treatment. In the first instance, the SEM associated with the mean fibre number indicates considerable variation in fibre number among animals (Table 3). This variation makes it difficult to demonstrate statistically-significant differences. Results from Bedi *et al.* (1978) demonstrate the problem. They reported an 18% decrease in fibre number in rats malnourished through gestation and suckling; however, their observations were not statistically significant due to a large SEM. In the second instance, the selection of a muscle may alter the conclusions about fibre number. For example, if only the fibre number of the plantaris (Table 3) were measured, fibre number would be interpreted as a constant value, whereas measurement of fibre number in the EDL reveals a significant decrease.

The limited information concerning the biochemical estimate of cell number and size serve to verify previous reports (Winick & Noble, 1966; Spence & Hansen-Smith, 1978). The effect of dietary restriction is to slow or halt the normal growth-related increases in DNA (Table 4).

Maintenance of cellular growth of skeletal muscles is dependent upon the severity of the nutritional restriction. Previous investigators have reported that restriction of dietary protein or energy would halt the growth-related increases in DNA (Hill *et al.* 1970; Howarth, 1972; Dickerson & McAnulty, 1975; Spence & Hansen-Smith, 1978). Summarizing these earlier reports, it appears that restriction of protein to a level less than or equal to a 60 g casein plus 3 g methionine/kg diet or restriction of total energy to less than 50% of normal, will cause cessation of nuclear proliferation in skeletal muscles. At intakes above these levels the reduction of nuclear number appears to be roughly proportional to the severity of the dietary restriction.

The dietary restriction caused a decrease in protein:DNA, an estimate of cell size, in the plantaris and EDL but not in the soleus. Thus, during severe weight loss, the body appears to be able selectively to maintain certain muscles such as the soleus.

Comparison of the anatomical and biochemical estimates of muscle cell size reveals that

the values are in reasonable agreement. The percentage decrease in protein:DNA is similar to the percentage loss of muscle weight, while fibre cross-sectional area, $\pi \times (\text{diameter}/2)^2$, decreased to a greater extent than muscle weight. This suggests that the majority of the weight lost is derived from the muscle fibres and not from extracellular components of the muscles. Mendes & Waterlow (1958) and more recently Spence & Hansen-Smith (1978) reported that during restriction of protein and energy the amount of muscle collagen remained constant or even increased while the non-collagenous protein decreased. These results imply that loss of protein from muscles is primarily derived from the loss of fibre protein.

Comparison of the anatomical and biochemical estimates of cell number reveal that the number of muscle fibres decreases during restricted food intake (Table 3) while the number of muscle nuclei remains relatively constant (Table 4). Earlier we reported (Layman *et al.* 1980) a similar inconsistency in these values during normal growth. During growth we found a decrease in the number of muscle fibres in each of four muscles examined. It is of interest to note that the growth-related decrease in fibre number is sufficient to account for the 5% loss of fibres in the plantaris in the present study and subtracting out this age effect from the soleus reduces the percentage change due to dietary restriction to 22%. These results indicate that in skeletal muscle, the physiological significance of the 'cell' as measured by DNA is not the same as that of the anatomical unit. In an attempt to explain the inconsistency, we postulated a mechanism of fibre fusion (Layman *et al.* 1980). We suggested that upon reaching some critical size muscle fibres coalesce, thus maintaining a larger fibre and also maintaining nuclear number. While this hypothesis appears to be consistent with all available information, no direct evidence is available to support it. However, recent reports examining increases in fibre number due to dystrophy-induced or exercise-induced hypertrophy have presented convincing evidence for splitting of muscle fibres (Swash *et al.* 1977; Vaughan & Goldspink, 1979; Gonyea, 1980). Thus the reverse of this process, fusion of fibres, appears reasonable.

Changes in fibre number and nuclear number have been investigated in three skeletal muscles after severe dietary restriction during postweanling growth of the rat. Nutritional restriction, sufficient to cause body-weight loss, was found to halt increases in nuclear number and to decrease the number of muscle fibres. We have also found that muscle fibres lost during postweanling dietary restriction can be regained upon refeeding (Hegarty & Kim, 1980). Presently, muscle fibre number is assumed to relate to growth potential. However, this assumption makes the relationship of nuclear number to growth potential unclear. Winick & Noble (1966) have presented evidence that the failure to increase DNA during this period of growth will result in a permanent decrease in DNA for these tissues and a decreased growth potential. Since fibre number is not permanently decreased during postweanling nutritional restriction it is suggested that after weaning DNA and not fibre number (cell number) is controlling the growth potential of rat skeletal muscle.

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