A critical period for formation of secondary myotubes defined by prenatal undernourishment in rats

S. J. WILSON, J. J. ROSS and A. J. HARRIS*

The Neuroscience Centre and Department of Physiology, University of Otago Medical School, PO Box 913, Dunedin, New Zealand

* To whom reprint requests should be sent

Summary

Rats fed a restricted diet during gestation and lactation gave birth to pups with about 60% of the normal birthweight. Maintaining the undernutrition after birth reduced the rate of growth of the pups so that their body weights were only 40% of control at PN7. Soleus and lumbrical muscles in these animals had reduced numbers of muscle fibres, and quantitative examination of embryonic muscles revealed that this was due solely to a decreased formation of secondary myotubes; the number of primary myotubes remained normal. Undernutrition did not affect the number of motoneurones surviving normal developmental death. Restoration of normal dietary intake on E21, one day before birth, did not correct the deficit in muscle fibre numbers in soleus muscles examined when the animals reached one month of age. Development of the lumbrical muscle lags behind the soleus and unrestricted feeding from E21 onwards allowed a normal number of fibres to develop from this time on, although the initial deficit was never restored. These experiments define a critical period in muscle development during which the potential maximum number of secondary myotubes is determined.

Key words: prenatal undernutrition, skeletal muscle, secondary myotubes, critical period, lumbrical muscle, soleus muscle, rat embryos.

Introduction

The number of fibres in a muscle depends both on genetic (Byrne et al. 1973; Stickland & Handel, 1986) and environmental circumstances. Muscle fibre numbers in young mammals are reduced following undernutrition of the mother during gestation and lactation (humans: Montgomery, 1962; sheep: Watland & Cassens, 1973; rats: Bedi et al. 1982) and also in the smallest animals within a litter of young (pigs: Hegarty & Allen, 1978; Wigmore & Stickland, 1983). This effect cannot be rectified by subsequent nutritional rehabilitation (Bedi et al. 1982). Mammalian muscle fibre numbers are not permanently affected by periods of undernutrition after weaning (pigs: Stickland et al. 1975; mice and hamsters: Goldspink & Ward, 1979; rats: Bedi et al. 1982).

Muscle development is biphasic (Kelly & Zacks, 1969; Ontell & Dunn, 1978). Myotubes form by fusion of mononucleate myoblasts, with primary myotubes forming first and providing a 'cellular skeleton' (Kelly, 1983) for the later development of secondary myotubes. Secondary myotubes initially form near the midpoint of a primary myotube, beneath the basal lamina (Ontell & Dunn, 1978). They then grow longitudinally and eventually connect with the muscle tendons and separate from the primary myotube. Secondary myotube numbers are sensitive to environmental variables such as paralysis or denervation (Harris, 1981; McLennan, 1983). Primary myotube formation, by contrast, has so far proved refractory to experimental manipulation (Harris, 1981; Wigmore & Stickland, 1983; Ross et al. 1987b).

In this study, we ask whether the reduction in muscle fibre number that follows maternal undernutrition is due solely to modulation of the number of secondary myotubes that form. To gain further understanding of the events involved in initiation of formation of secondary myotubes, we also assess the capacity of muscles at different stages of development to 'catch up' after the undernutrition-imposed slowing of the rate of myotube formation. This is done by restoring a normal diet to the mothers one day prior
to birth, a time when 40% of the secondary myotubes would normally have been generated in soleus and 20% in lumbrical muscles. Our results enable us to define a critical period in muscle development during which secondary myotubes must be determined to form if they are ever to be present in the muscle.

Materials and methods

Adult male and virgin female white Wistar rats (200–250 g) were mated in wire-bottomed cages. The day a copulation plug was found was designated day zero of the pregnancy and the female was caged singly from that time.

Pregnant females were allowed free food for the first 2 days of gestation, and then assigned to control, undernourishment and rehabilitation groups. Control females were allowed food (standard rat pellets) ad lib throughout gestation and lactation. Undernourished rats were kept on a restricted ration of about 30% of the control food intake. Rats in the rehabilitation group were kept on this regime until day 21 of gestation (E21), one day before parturition, from which time they were allowed food ad lib. All animals had free access to water. Body weights of mothers and young were recorded each morning. Litter sizes were standardized to eight pups at birth.

Control animals were taken at E17 to count soleus muscle primary myotubes, at E20 and at postnatal days 7 (PN7) and 28 (PN28). Undernourished animals were examined at E20 and PN7, and rehabilitated animals at PN28. To obtain the fetuses, the mothers were heavily anaesthetized with ether and fetuses from near the bottom of the uterine horns removed, placed on ice to maintain anaesthesia and perfused through the heart with warmed fixative. The fixative contained 1% paraformaldehyde, 1-25% glutaraldehyde and 0-5mM-CaCl₂ in 0-14M-Hepes, with SOi.u.ml⁻¹ heparin. Two embryos, of undetermined sex, were normally taken from each litter. Postnatal tissue was taken from male rats anaesthetized with ether and perfused through the heart with fixative containing 1% paraformaldehyde, 1% glutaraldehyde, 0-4 mM-CaCl₂, 0-03M-glucose in 0-1M-phosphate buffer with 50i.u.ml⁻¹ heparin. Two embryos, of undetermined sex, were normally taken from each litter. Postnatal tissue was taken from male rats anaesthetized with ether and perfused through the heart with fixative containing 1% paraformaldehyde, 1% glutaraldehyde, 0-4 mM-CaCl₂, 0-03M-glucose in 0-1M-phosphate buffer with 50i.u.ml⁻¹ heparin. Up to three siblings were taken from each litter.

Following perfusion, the soleus and IVth lumbrical muscles and the L4 ventral roots were dissected and immersed in fixative for a total of 4h. Tissues from both sides of the animal were normally retained. The tissues were postfixed in osmium tetroxide and stained en bloc in uranyl acetate, dehydrated and embedded in TAAB epoxy resin. Ultrathin transverse sections (≈90nm) were cut through E20 soleus and lumbrical and PN7 lumbrical muscles at the midbelly endplate-containing region and through the ventral nerve roots just proximal to the dorsal root ganglion. The sections were mounted on single-slot Formvar-coated copper grids, stained with uranyl acetate and lead citrate and viewed and photographed with a Philips 410 electron microscope. Semithin sections (1 µm) from PN7 soleus and PN28 lumbrical and soleus muscles were mounted on glass slides, stained with methylene blue and azure II, and viewed and photographed with light microscopy. Photomontages of the entire muscle and nerve sections were produced, and all of the fibres counted.

Results

Undernutrition

Body weights

At birth, the undernourished pups had a 42% deficit in body weight compared to controls. Continued undernutrition of the mother to PN7 resulted in a 61% deficit in the body weights of the pups compared to age-matched controls.

Muscle fibre numbers

Soleus muscles from control animals were examined at E17 to count primary myotubes. There were 84 ± 1-7 (n = 4) primary myotubes in this muscle. At E20 we did not attempt to distinguish primary and secondary myotubes. At this time, there were 914 ± 38 (n = 4) muscle fibres in the control soleus and 649 ± 25 (n = 7) muscle fibres in the undernourished soleus muscles (Fig. 1). This is a 29% reduction in soleus muscle fibre number as a result of prenatal undernutrition.

By E20, the IVth lumbrical muscle normally has its full complement of primary myotubes and has been generating secondary myotubes for approximately 1 day (Ross et al. 1987a), and these two populations are readily distinguishable with electron microscopy (Fig. 2A). Primary myotubes are large cells containing abundant well-organized myofilaments. Secondary myotubes are small myofilament-containing cells...
Fig. 2. Electron micrographs of E20 lumbrical muscles from control and undernourished rat fetuses. (A) Control muscle. Clusters of myotubes including a primary myotube ($1^\circ$), one or two secondary myotubes ($2^\circ$), and mononucleate cells ($mn$) are enclosed within a basal lamina. (B) Undernourished muscle. Primary myotubes predominate with only an occasional secondary myotube present. Calibration bars, 2 $\mu$m.
lying under the same basal lamina and sometimes interdigitating with primary myotubes. The numbers of primary myotubes were the same in undernourished (110 ± 2, n = 7) and control (109 ± 2, n = 7) lumbrical muscles (P > 0.7). There were, however, very few secondary myotubes in the undernourished (13 ± 1) as compared to the control lumbricals (78 ± 5) (P < 0.001) (Figs 2B, 3), so that the total number of muscle fibres was 34 % less than controls (Fig. 4).

The undernourished E20 lumbrical muscles were less mature than the controls. In the controls, primary myotubes were separate and most had one or more associated secondary myotubes, in varying stages of maturation (Fig. 2A). In the undernourished muscles, by contrast, the primary myotubes often had not separated and remained in groups of two or occasionally three, and were sometimes linked by gap junctions. Secondary myotubes, in addition to being reduced in number, were usually small and closely interdigitated with the primary myotube. There was rarely more than one secondary myotube associated with each primary myotube (Fig. 2B). This level of maturity is similar to that of normal lumbrical muscle at E19 (Ross et al. 1987a).

Mononucleate cells lying within the basal lamina, which may include presumptive fibroblasts as well as myoblasts, were counted in the single midbelly cross-sections of soleus and lumbrical muscles at E20. There was no difference between the number of these cells in the undernourished and control lumbrical muscles (Fig. 3). In soleus muscles, there was a 27 % diminution in mononucleate cell number in the muscles from undernourished animals, but this was barely statistically significant (0.05 < P < 0.1) and matched the reduction in muscle fibre number.

When the restricted diet was continued during the first week after birth there was a persisting deficit in muscle fibre number in the pups (Fig. 1). At PN7, control soleus muscles had 2789 ± 15 muscle fibres (n = 3) and undernourished muscles had 2255 ± 54 (n = 5), a 19% reduction (P < 0.001).

Undernourished lumbrical muscles at PN7 had 25 % fewer fibres than controls (727 ± 12, n = 8 versus 974 ± 18, n = 8; P < 0.001) (Fig. 4), and 28 % fewer mononucleate cells, matching the reduction in fibre number.

**Ventral root axon numbers**

In order to see whether the reduction in secondary myotube formation might be secondary to an increase in motoneurone death, L4 ventral root axons were counted at E20, PN7 and PN28. Promyelinated fibres were counted at E20 and, with the data from one animal omitted, there was no difference between control and undernourished animals (2380 ± 30, n = 7 versus 2553 ± 26, n = 6; P > 0.25). The exception was an undernourished fetus whose ventral roots on both sides had about 30 % more axons than any other, including its siblings. These high numbers are comparable with those found in normal E18 fetuses (Ross et al. 1987a) and may be due to delayed motoneurone death in this animal. The muscle fibre counts from
Nutritional rehabilitation

Body weights
When rats were undernourished during pregnancy but given free access to food from E21 their young had birth weights 31% less than controls (19% greater than the continually undernourished group). This deficit persisted through lactation.

Muscle fibre numbers
Muscles from animals in the rehabilitation group were examined at PN28. Soleus muscles had the same number of fibres as in muscles from underfed animals examined at PN7, significantly less than controls (Fig. 1). Thus, although rehabilitation was effective in increasing body weight, it did not increase the number of soleus muscle fibres able to be generated.

In PN28 lumbrical muscles, however, the number of muscle fibres in muscles from rehabilitated animals, while still less than control (863 ± 11, n = 8 versus 979 ± 23, n = 6; P < 0.001), was significantly greater than in the undernourished animals at PN7 (P < 0.001) (Fig. 4). Since the normal number of primary myotubes had already been generated at E20 in both control and undernourished lumbrical muscles, these extra fibres must have all been of secondary myotube origin. During the period E20 to PN28, the rehabilitated animals generated 740 secondary myotubes and the controls 792. Thus the number of secondary myotubes generated during this period was not substantially less in rehabilitated than in control animals, but the initial fibre deficit was not made up.

Ventral root axons
At PN28 the control L4 ventral roots contained 1950 ± 112 myelinated axons (n = 6) and ventral roots from rehabilitated animals contained 1778 ± 78 axons (n = 10). These numbers are not significantly different (P > 0.2). We also assayed subclasses of axon according to fibre size and found no significant differences.

Discussion
Our results show that production of secondary myotubes, but not of primary myotubes, is sensitive to prenatal undernutrition. In addition, an experiment with nutritional rehabilitation revealed that secondary myotube numbers could be recovered, but success in this manoeuvre depended on the stage of development of the muscle. From these results, we suggest that there is a critical period in development during which the maximum number of adult muscle fibres is determined.

These results confirm and extend previous observations. Although there have been many studies of the effects of nutritional deprivation on muscle growth and muscle fibre number, few of these have involved undernourishment during the period of muscle fibre generation. We severely undernourished pregnant rats (30% of normal food intake) from day 2 of gestation onwards, and examined their offspring 2 days before and one week after birth. The muscles we studied, the soleus and IVth lumbrical, had 19–34% deficits in fibre number, as determined by counting every fibre in the muscle cross-section. This deficit is comparable to that found by Bedi et al. (1982) who counted soleus and extensor digitorum longus muscle fibres (19% and 21% reductions, respectively) in the adult offspring of does undernourished during gestation and lactation.

By examining lumbrical muscles on E20, we show that only secondary myotube generation is affected by prenatal undernutrition. This differential response was already suggested by Wigmore & Stickland (1983) who in a semiquantitative light microscope study of fetal pigs found that semitendinosus muscles in the smallest animals had fewer secondary myotubes but the same number of primary myotubes as those in their larger littermates. Fetal growth retardation is probably a consequence of prenatal malnutrition due to the position of the animal within the uterine horn (McLaren, 1965).

A critical period for secondary myotube formation
Our most interesting finding is that there is a critical period for initiating formation of secondary myotubes and that this period may precede the actual generation of the myotube. Although only about 40% of soleus muscle secondary myotubes would normally have formed by E21 (calculated by extrapolation), refeeding undernourished animals from that time did not correct the depression in their capacity to generate secondary myotubes. The lumbrical muscle is developmentally about 1 day behind the soleus muscle and only 19% of the secondary myotubes have developed by E21 (Ross et al. 1987a). Refeeding after previous undernutrition restored almost the full myogenic capacity of this muscle, but there was no compensation for myotubes not formed prior to refeeding.
Role of innervation in regulating muscle fibre numbers

Evidence from experiments on frogs (Ferns & Lamb, 1987) and rats (Ross et al. 1987b) shows a stoichiometric relation between muscle innervation and secondary myotube generation. Accordingly, it was important to ask whether undernutrition affected muscle innervation. We found no evidence for excess motoneurone death in undernourished animals. We cannot, however, comment on whether undernourished motoneurones formed fewer than their normal number of terminals, which in normal development form in advance of the muscle fibres they will eventually innervate (Harris & McCaig, 1984).

Mechanism of the effect of undernutrition

Fairly severe undernutrition was required before we saw any effect on muscle fibre numbers. In a preliminary experiment, holding animals at constant weight during pregnancy had no effect on muscle fibre numbers in their offspring. Beerman (1983) found a reduction to 50% of normal food intake from the 8th day of pregnancy onwards in rats did not affect muscle fibre number in the offspring, although it had a long-lasting effect on muscle growth.

We also did not see the marked reduction in mononucleate cell numbers in the midbelly region of the muscle that accompanies the halt in secondary myotube production produced by fetal denervation (Ross et al. 1987b). The reduction we saw after fetal malnutrition was such that the ratio of mononucleate cells to total fibre number remained constant. This implies that the rates of myoblast proliferation and fusion are both reduced in the muscles of the undernourished animals, even if the number of myoblasts fusing to form each myotube remained constant (Penney et al. 1983; McLennan, 1987).

As the number of secondary myotubes that form in a muscle is well regulated (Ross et al. 1987a) and very much less than the number of fusion-competent cells that are present throughout development, it appears that secondary myotube formation is initiated by a special cell fusion event associated with the presence of a nerve terminal (Ross et al. 1987b). Once the nascent secondary myotube exists, it can grow by accepting fusion from myoblasts that come in contact with it. It is likely that the effect of undernutrition is, directly or indirectly, to reduce the number of the subpopulation of myogenic cells which participate in the initiating event, thereby permanently limiting secondary myotube numbers.

This work was supported by the New Zealand Medical Research Council, the Vernon Willey Trust and the Muscular Dystrophy Association. We thank K. S. Bedi for advice on the regime for undernourishment.

References


Critical period in skeletal muscle development


(Accepted 19 January 1988)