Postnatal regulation of myosin heavy chain isoform expression and metabolic enzyme activity by nutrition

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Development of muscle is critically dependent on several hormones which in turn are regulated by nutritional status. We therefore determined the impact of mild postnatal undernutrition on key markers of myofibre function: type I slow myosin heavy chain (MyHC) isoform, myosin ATPase, succinate dehydrogenase and α-glycerophosphate dehydrogenase. *In situ* hybridization, immuno-cytochemistry and enzyme histochemistry were used to assess functionally distinct muscles from 6-week-old pigs which had been fed an optimal (6 % (60 g food/kg body weight per d)) or low (2 % (20 g food/kg per d)) intake for 3 weeks, and kept at 26°C. Nutritional status had striking muscle-specific influences on contractile and metabolic properties of myofibres, and especially on myosin isoform expression. A low food intake upregulated slow MyHC mRNA and protein levels in *rhomboideus* by 53 % ($P < 0.01$) and 18 % ($P < 0.05$) respectively; effects in *longissimus dorsi*, *soleus* and diaphragm were not significant. The oxidative capacity of all muscles increased on the low intake, albeit to varying extents: *longissimus dorsi* (55 %), *rhomboideus* (30 %), *soleus* (21 %), diaphragm (7 %). Proportions of slow oxidative fibres increased at the expense of fast glycolytic fibres. These novel findings suggest a critical role for postnatal nutrition in regulating myosin gene expression and muscle phenotype. They have important implications for optimal development of human infants: on a low intake, energetic efficiency will increase and the integrated response to many metabolic and growth hormones will alter, since both are dependent on myofibre type. Mechanisms underlying these changes probably involve complex interactions between hormones acting as nutritional signals and differential effects on their cell membrane receptors or nuclear receptors.

Energy balance: Myosin heavy chain isoforms: Respiratory enzymes: Skeletal muscle development: Undernutrition

Skeletal muscle plays a key role in determining nutrient oxidation rates, is the main peripheral site of insulin action and is essential for locomotion, postural maintenance, breathing and thermogenesis. Defects in its normal development can thus profoundly influence metabolic and contractile function. During muscle development, the commitment of precursor cells to form myoblasts in early embryonic life is followed by differentiation into myotubes and maturation into myofibres. The structural and functional diversity of skeletal muscles reflects an intricate combination of contractile and metabolic attributes (Rowlerson, 1994). The ATPase activity of the myosin heavy chain (MyHC) isoforms expressed within each fibre is a key factor in determining contractile ability (Schiaffino & Reggiani, 1994; Harridge *et al.* 1996), while the relative proportions of mitochondria and respiratory enzymes determine the metabolic properties of the muscle (Dauncey & Gilmour, 1996; Harrison *et al.* 1997). The four important

myofibre types occurring in mature mammalian muscle are type I slow oxidative, type IIA fast oxidative–glycolytic, type IIX fast oxidative–glycolytic and type IIB fast glycolytic.

The sequential expression of MyHC isoforms prenatally, i.e. embryonic → fetal → neonatal → adult (type I slow and type II fast), can be followed by further transitions between adult isoforms postnatally, i.e. type I ↔ IIA ↔ IIX ↔ IIB (Schiaffino & Reggiani, 1994; Lefaucheur *et al.* 1998; McKoy *et al.* 1998). Contractile activity and hormonal status play a central role in regulating these transitions, and the effects are dependent on stage of development (Dauncey & Gilmour, 1996; Goldspink, 1996). Prenatally, for example, thyroid hormones (TH) induce myoblasts to exit the cell cycle and form myotubes, whereas postnatally they stimulate conversion of type I to type II MyHC isoforms (d'Albis & Butler-Browne, 1993) and increase the abundance of mitochondria and their respiratory

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enzymes (Herpin *et al.* 1996). Other hormones implicated in muscle development and differentiation between MyHC isoforms include growth hormone (Ayling *et al.* 1992), glucocorticoids (Polla *et al.* 1994), insulin (Cotter & Cameron, 1994) and insulin-like growth factors (Florini & Ewton, 1992). Nutrition, and especially level of energy intake, has marked effects on hormonal status. Postnatally, a low food intake that is not severe enough to prevent growth is associated with reduced plasma TH, insulin and insulin-like growth factor-I levels and fewer muscle TH receptors, and increased plasma growth hormone and gluco-corticoid levels and muscle growth hormone receptor expression (Dauncey *et al.* 1989, 1994; Harrison *et al.* 1996*b*; Morovat & Dauncey, 1998). The possibility there-fore arises that energy status via hormones acting as nutri-tional signals plays an important role in regulating muscle development (Dauncey, 1997).

It is well-recognized that undernutrition influences muscle hyperplasia and hypertrophy, with the effects being dependent on stage of development (Stickland *et al.* 1975; Ward & Stickland, 1993). However, relatively little is known about the influence of nutrition on muscle develop-ment at the molecular level, and specifically on myofibre differentiation and MyHC isoform expression. The effects of nutrition are probably dependent on stage of development and muscle type. Perinatally, undernutrition delays matura-tional switching from embryonic–neonatal to adult MyHC in rat diaphragm (Brozanski *et al.* 1991). In adults, the major effect of energy deficiency is a reduction in type II fibre size, whereas the size of type I fibres is selectively preserved (Schantz *et al.* 1983). Postnatally, by contrast, under-nutrition induces a muscle-specific increase in proportion of type I slow fibres (Harrison *et al.* 1996*a*). Whether these changes are related to changes in MyHC gene expression is not known. We have therefore tested the hypothesis that postnatal energy status regulates the type I slow MyHC isoform at both the transcriptional and translational levels. The type I isoform was chosen because it provides key information on muscle development and function. The aim was to investigate physiological changes associated with mild undernutrition, rather than pathological changes associated with starvation. Therefore, a reduction in energy intake, which is known to have a major impact on hormonal status but nevertheless is not severe enough to prevent growth, was studied. A range of morphologically and functionally distinct skeletal muscles was investigated and, in order to provide a comprehensive view of the effects of nutrition on the contractile and metabolic properties of muscle, an assessment was also made of the role of energy intake in modulating the metabolic activity of myofibres postnatally.

Materials and methods

Animals, study design and tissue sampling

Studies were undertaken in the young pig, a species which makes a particularly good developmental, nutritional, hormonal and metabolic model for the human infant (Tumbleson & Schook, 1996). Because of the need for precise control of food intake, animals were investigated immediately after weaning, at 3 weeks of age. Feeding regimens were selected to allow investigation of physio-logical changes associated with mild undernutrition, rather than pathological changes associated with starvation, and food intakes were based on our previous experience of energy balance in young growing animals.

Eight pairs of littermate pigs of the Large White breed were investigated. At 3 weeks, animals were housed in pairs for the first 1–2 d, and then placed in separate pens within the same room to allow careful control of food intake. To avoid any major impact of environmental temperature on energy balance, animals were kept at 26°C, i.e. within the zone of thermal neutrality. They were weighed three times per week, immediately before feeding. The food (Ultra-Wean, Dalgety, Bristol, UK) was provided *ad libitum* for the first 2 d after which time food intake was controlled. One littermate was provided with a high energy intake (6 % (60 g food/kg body weight per d)) while the other received a low energy intake (2 % (20 g food/kg body weight per d)), provided as two meals per d at 09.30 and 16.30 hours. A level of 6 % was chosen for the high intake because it is slightly below the *ad libitum* intake of approximately 8 % in young pigs, and enables optimal growth. The 2 % intake was chosen because it provides enough energy to enable a low rate of growth and is not severe enough to prevent growth. The food contained 14 kJ gross energy/g wet weight, and comprised 32 % carbohydrate, 22·5 % protein, 5·5 % fat, 3·5% fibre and 6 % ash, with added vitamins and minerals. Water was freely available and lighting was on from 09.00 to 21.00 hours daily. The treatments were maintained for 3 weeks, during which time all animals grew, although at very different rates. A 3-week period of study was chosen because we did not want to investigate the immediate response to a change in food intake and therefore needed to wait until differences had become established.

At 6 weeks the animals were killed for tissue sampling, at 15–16 h after the last meal, as described previously (Harrison *et al.* 1996*a*). All animal protocols were approved by the UK Home Office. The timing of tissue sampling was standardized because several hormonal and metabolic variables which affect muscle development are themselves influenced by feeding (Dauncey *et al.* 1994; Morovat & Dauncey, 1998). The following morphologically and functionally distinct muscles were dissected rapidly, taking care to sample muscles from the same relative point with respect to both depth and distance from origin: *longissimus dorsi* (*l. dorsi*), a predominantly fast-twitch oxidative–glycolytic dorsal-lumbar muscle, used in rapid movement; *rhomboideus*, a mixed slow- and fast-twitch oxidative–glycolytic dorsal-interscapular muscle, which may have roles in both postural maintenance and thermoregulation; *soleus*, a slow-twitch oxidative postural muscle of the hind-leg; and diaphragm, a mixed slow- and fast-twitch oxidative–glycolytic muscle. Samples of 1 cm^3 were mounted on cork blocks, surrounded by Cryo-m-bed (Bright Instrument Company Ltd, Cambridge, Cambs., UK), and frozen immediately in isopentane cooled in liquid N$_2$, before storage at −70°C. Ten serial 10 μm cross-sections were later cut on a cryostat at −22°C, and used for measurement of type I slow MyHC mRNA expression by *in situ* hybridization, type I slow MyHC protein level by immunocytochemistry, and myosin

ATPase (mATPase) and respiratory enzyme activities by histochemistry.

In situ *hybridization*

In brief, frozen sections were fixed onto 3-aminopropyl-triethoxysilane-coated slides using paraformaldehyde. Through a series of washes and proteinase K treatment, the tissue was permeabilised to allow access of the probe to the mRNA transcripts. Sections were hybridised with a ^{35}S-UTP labelled β-MyHC (type I slow myosin) riboprobe (kindly donated by Dr K. C. Chang, University of Glasgow, Scotland, UK). The probe had been cloned into a pBlue-script plasmid and consisted of the 5′ end of a polymerase chain reaction-derived β-MyHC cDNA of 340 nucleotides in length, covering exons 1 to 3 and part of exon 4 (Chang *et al.* 1993). The plasmid was linearised and the probe

transcribed using T7 for anti-sense, and T3 for the sense direction which was used routinely as a negative control. After over-night hybridization at 55°C, the unbound and non-specifically bound probe was removed by ribonuclease treatment and a series of washes. The slides were dehydrated through an ethanol series, air-dried, coated with a 1:2 dilution of Ilford K5 photoemulsion (Ilford Imaging UK Ltd, Mobberley, Ches., UK) and exposed for 5 d in a light-proof box containing silica gel. The slides were developed in Ilford LC29 developer (Ilford Imaging Ltd), fixed, dried, counterstained and mounted ready for analysis.

Immunocytochemistry

Myofibres expressing type I slow MyHC protein were identified using a slow MyHC (type I)-specific mono-clonal antibody (Clone no. MHCs, Biogenesis Ltd,
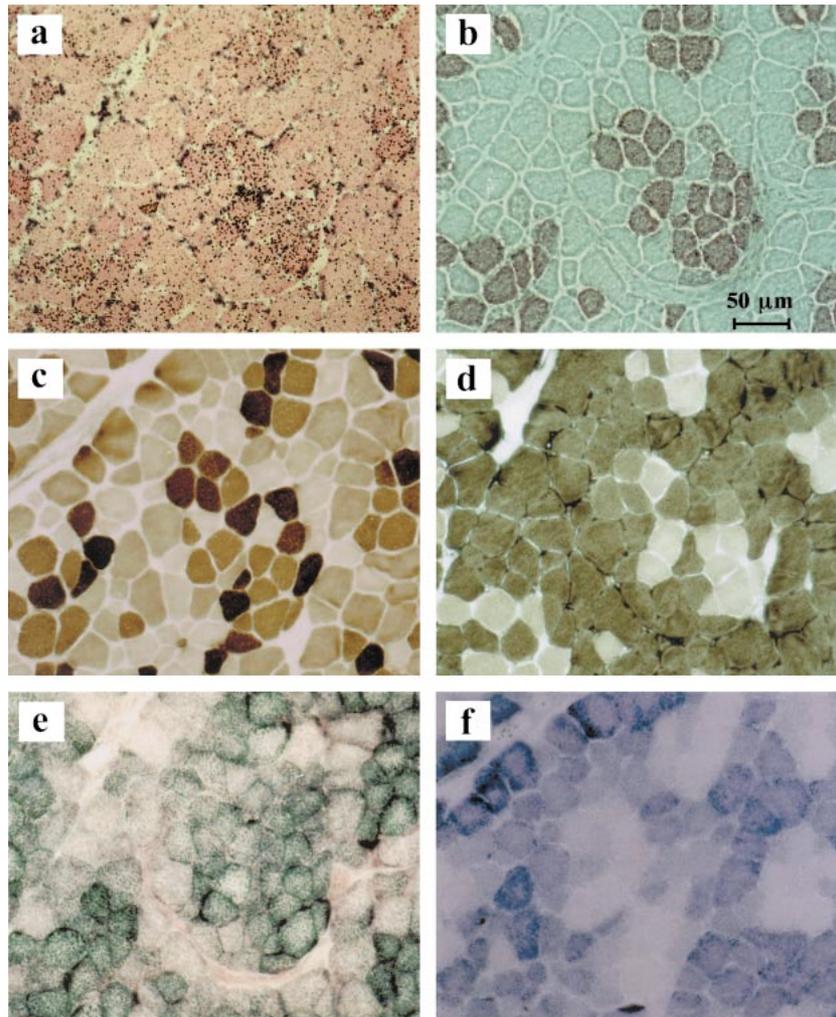

Fig. 1. Assessment of myofibre genotype and phenotype in postnatal pig diaphragm muscle. Assessment was made in 10 μm serial frozen sections of (a) type I slow myosin heavy chain (MyHC) mRNA expression, by *in situ* hybridization; (b) type I slow MyHC protein expression, by immunocytochemistry; (c) type I slow myofibres, by mATPase activity at a pre-incubation pH of 4·45; (d) fast type II myofibres, by mATPase activity at a pre-incubation pH of 9·4; (e) oxidative myofibres, by succinate dehydrogenase histochemistry; (f) glycolytic activity, by menadione-linked α-glycerophosphate dehydrogenase histochemistry.

Poole, Dorset, UK). The antibody had been raised in the mouse using native type I myosin from rabbit *soleus* as an immunogen. In brief, frozen sections were placed on 3-aminopropyltriethyoxysaline-coated slides, fixed in 4% paraformaldehyde for 10 min, and washed in Tris-buffered saline for 20 min. Sections were blocked with normal horse serum for 30 min and incubated with the primary MyHC (type I) antiserum diluted 1 : 50 in normal horse serum for 1 h at room temperature. After 3×10 min washes in Tris-buffered saline–Tween, sections were incubated with a peroxidase-labelled anti-mouse IgG antibody (Vector Laboratories Ltd, Peterborough, Cambs., UK) diluted 1 : 200 in normal horse serum. Sections were washed, incubated with an avidin–biotin complex (ABC reagent; Vector Laboratories Ltd), rinsed and incubated in 0·03% H_2O_2 in Tris-buffered saline + 1 mg diaminobenzidine/ml until the stain was seen to develop. After dehydration through ethanol and xylene, the sections were mounted in a xylene-based mounting medium.

Enzyme histochemistry

The activity of mATPase was used to assess myofibre types, as described previously (Harrison *et al.* 1996*a*). The method is based on the ability of myosin to hydrolyse ATP in the presence of Ca^{2+} at pH 9·4, coupled with the stability of mATPase after pre-incubation at different pHs. In brief, air-dried serial sections were preincubated at acidic or alkaline pH for 5 min, and the sections were rinsed and incubated in buffer containing ATP and Ca^{2+}. The phosphate ions released were precipitated as insoluble calcium phosphate and converted via a series of exchanges to insoluble black cobalt sulphide. The intensity of colouration is proportional to mATPase activity; fibres that have retained their activity stain dark brown–black, while those whose activity was destroyed by the pre-incubation pH are pale brown–white. Several fibre types have been identified using this method, including type I slow oxidative, type IIA fast oxidative–glycolytic, type IIB fast glycolytic and type IIC oxidative (Harrison *et al.* 1996*a*). For adult porcine muscle, pre-incubation acidic pHs of 4·2, 4·4 and 10·4 allow clear distinction between fibre types (Lefaucheur *et al.* 1995).

The presence of succinate dehydrogenase activity was used as a marker of fibres with oxidative capacity as described previously (Dauncey & Ingram, 1988). In brief, air-dried frozen sections were incubated at 37°C in medium containing 125 mM-sodium succinate, 0·1% Nitroblue Tetrazolium (w/v), 5 mM-MgCl₂, 50 mM-Tris-HCl, pH 7·4, for 1 h. Sections were fixed in formol saline for 10 min, washed in water, and mounted in glycerol jelly.

The presence of α-glycerophosphate dehydrogenase activity was used as a marker of fibres with glycolytic activity as described previously (Harrison *et al.* 1997). In brief, air-dried frozen sections were incubated at 37°C in medium containing 9·25 mM-α-glycerophosphate, 0·1% Nitroblue Tetrazolium (w/v), 0·02% menadione (w/v), 50 mM-Tris-HCl, pH 7·4, for 1 h. Sections were washed for 20 s in a sequence of acetone solutions (30%, 60%, 90%, 60% and 30%), washed in water and mounted in glycerol jelly.

Assessment of myofibre proportions and statistical analysis

Use of the five methods outlined earlier, on serial frozen sections of diaphragm muscle, is illustrated in Fig. 1. Assessment was made of the proportion of myofibres exhibiting the specific properties identified by each method. For *in situ* hybridisation and immunocytochemistry, fibres were recorded as expressing type I MyHC when the intensity of staining was significantly above the background level. For mATPase staining at an alkaline pre-incubation pH, fibres retaining their mATPase activity and thus staining brown–black were recorded as type II, and pale brown–white fibres were recorded as type I. For succinate dehydrogenase and α-glycerophosphate dehydrogenase activity, fibres were recorded as having either a high (++), intermediate (+) or low (−) level of activity, dependent upon the intensity of the staining. Our initial studies showed that the same results were obtained by two independent observers trained to the same level of expertise. In the definitive study, all measurements were made by one observer in order to reduce any potential variability to a minimum.

Within each muscle section, four random fields of view within a standard field of 119 000 μm^2 were selected, the numbers of specific fibres were counted with a Seescan A010 research-grade image analysis system (Seescan Ltd, Cambridge, Cambs., UK), and values were expressed as a percentage of the total fibre count. This number of randomly selected fields of view led to approximately 500 fibres being counted in total and was considered statistically appropriate for the following reason. Using χ^2 contingency table analysis, it was first established that there was little evidence for clustering of fibre types within the chosen muscles. In the
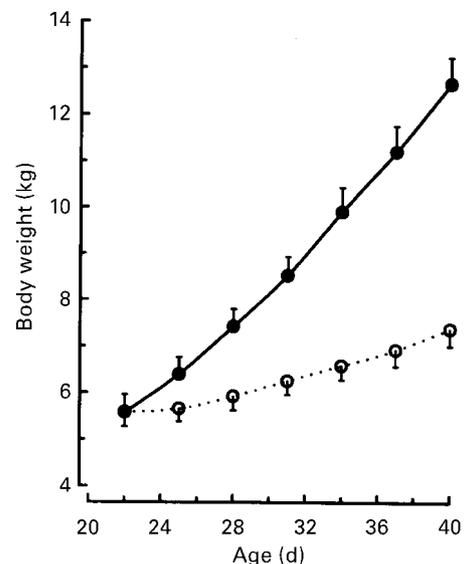

Fig. 2. Growth rates of animals during 3 weeks on a high or low energy intake. Within each littermate pair, one pig was provided with a high intake ((—●—), 6% (60 g food/kg body weight per d)) and the other a low intake (- - -○- - -), 2% (20 g food/kg body weight per d)). Values are means for eight littermate pairs with standard errors of the means represented by vertical bars.
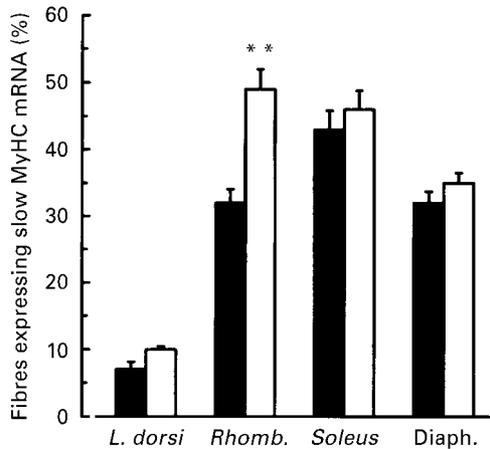
Fig. 3. Nutritional regulation of type I slow MyHC mRNA expression. Proportion of fibres expressing the type I slow MyHC gene in pigs on a high ((■), 6 % (60 g food/kg body weight per d)) or low ((□), 2 % (20 g food/kg body weight per d)) food intake, in *longissimus dorsi* (*l. dorsi*), *rhomboideus* (*rhomb.*), *soleus* and diaphragm (*diaph.*) muscles. Mean value was significantly different from the high-intake group, ** $P < 0.01$. Values are means for eight littermate pairs with standard errors of the means represented by vertical bars.

the relative error extremely small. Mean values of fibre type proportions for each muscle and both treatment groups are presented. Standard error of the mean was calculated using the 'nonbiased' or '$n-1$' method and Student's paired t test was used to test for statistical significance between the two treatment groups at the 5 %, 1 % and 0·1 % levels.

Results

Growth rates

Fig. 2 shows that animals on the high energy intake (6 %) grew very rapidly during the treatment period, reaching a mean body weight which was almost double that of their littermates on the low energy intake (2 %). It should be noted, however, that the low intake did not prevent growth and all animals grew throughout the study. At the start of investigation, body weights at 3 weeks of age were similar for all groups and close to 6 kg. By 6 weeks, mean body weights (kg) were 12·8 (SEM 0·6) and 7·2 (SEM 0·4) for the high- and low-treatment groups respectively. The overall effect of energy intake on animal growth was highly significant ($P < 0.001$).

absence of clustering, the standard error of an estimated proportion (p), where the true proportion is T using a sample size of *n*, is given by $\sqrt{(T(1-T)/n)}$, which takes the following values for *n* 500: T 0·5, SE (p) = 0·022; T 0·2, SE (p) = 0·018; T 0·05, SE (p) = 0·0097. Since fibre proportion was assessed using samples from *n* 6–8 animals, this SE would be reduced by a further factor of $\sqrt{6}$ or $\sqrt{8}$, making

Muscle-specific regulation of type I slow myosin heavy chain mRNA and protein expression

In situ hybridization revealed striking muscle-specific effects of postnatal nutrition on type I slow MyHC mRNA expression (Figs 3 and 4). In *rhomboideus*, the proportion of fibres expressing type I mRNA was on average 53 % greater
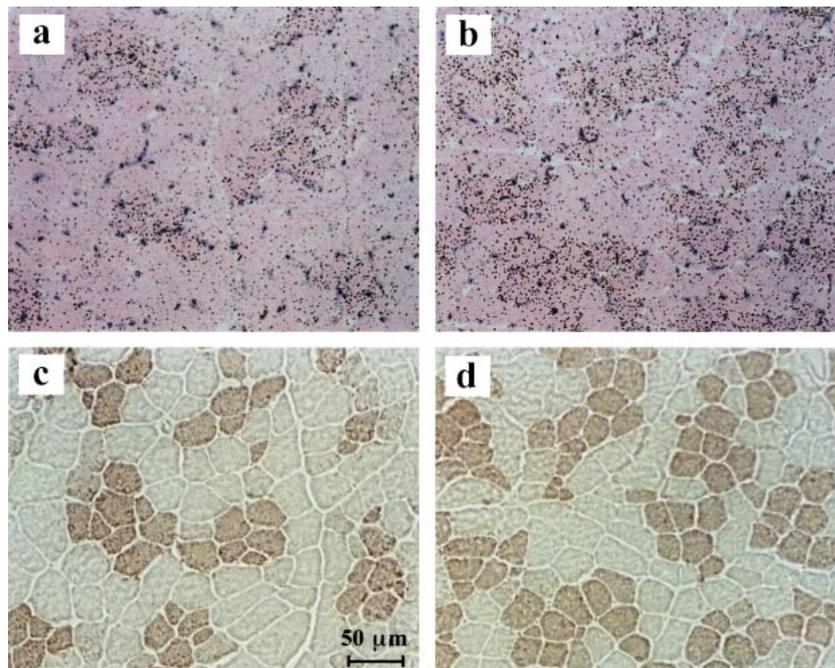

Fig. 4. Nutritional regulation of type I slow MyHC expression in *rhomboideus* muscle. Frozen 10 μm sections were assessed for (a), (b), mRNA expression or (c), (d), protein expression. Samples were from 6-week-old littermate pigs on either (a), (c), a high (6 % (60 g food/kg body weight per d)) food intake or (b), (d) a low (2 % (20 g food/kg body weight per d)) food intake.
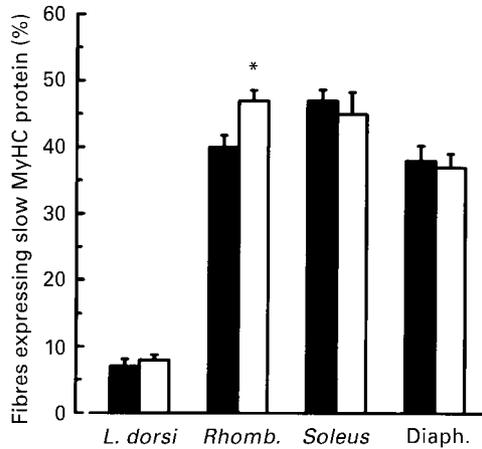
Fig. 5. Nutritional regulation of type I slow MyHC protein expression. Proportion of fibres expressing the type I slow myosin heavy chain protein in muscles from pigs on a high ((■), 6 % (60 g food/kg body weight per d)) or low ((□), 2 % (20 g food/kg body weight per d)) food intake. *L. dorsi, longissimus dorsi*; *rhomb., rhomboideus*; *diaph., diaphragm*. Mean value was significantly different from that of the high-intake group, * $P < 0.05$. Values are means for six littermate pairs with standard errors of the means represented by vertical bars.

in animals on the low compared with the high energy intake ($P < 0.01$). In *l. dorsi, soleus* and diaphragm, by contrast, the proportion of fibres expressing type I MyHC mRNA was only slightly greater in animals on the low compared with the high intake, and none of these comparisons was statistically significant.

Indirect immunoperoxidase staining demonstrated that nutrition also has muscle-specific effects on expression of type I MyHC protein. However, the effects were not as great as for type I MyHC mRNA (Figs 4 and 5). In *rhomboideus*, expression of the protein was on average 18 % greater in animals on the low than the high energy intake ($P < 0.05$).

Levels of type I MyHC protein in *l. dorsi, soleus* and diaphragm were not affected by diet.

Comparison between Figs 3 and 5 indicates that in animals on the 2 % intake the proportion of myofibres expressing type I MyHC mRNA was very similar to that at the protein level, for all muscle investigated. However, in animals on the 6 % intake the proportion of myofibres in *rhomboideus* which expressed type I MyHC mRNA was considerably lower than those expressing the protein, and this also tended to be the case for *soleus* and diaphragm. Taken together, these findings suggest that there is a striking influence of nutrition on type I MyHC expression in *rhomboideus* muscle at both the transcriptional and translational levels.

Assessment of myofibre type by ATPase histochemistry

Comprehensive evaluation of the technique for ATPase histochemistry showed that although it can be used to distinguish between type I slow and type II fast fibres postnatally, discrimination between fast sub-types is not possible. Use of mATPase histochemistry at acidic pre-incubation pHs was found to be unsuitable for fibre typing because, in muscles from the 6-week-old animals used in this study, acid lability was highly variable between myofibres, resulting in a gradient of staining (Fig. 1). In adult porcine muscle, by contrast, we were able to distinguish between types I, IIA, IIB and IIC when using an acidic pre-incubation pH (data not shown). At a pre-incubation pH of 9·4, clear discrimination between type I slow and type II fast fibres was possible in muscles from the young animals. In *rhomboideus*, the proportion of type I fibres was found to be 30 % greater in animals on the low than the high intake ($P < 0.05$). The other muscles examined showed no effect of nutrition on the proportion of type I fibres. Mean values (%) of type I fibres in *l. dorsi, rhomboideus, soleus* and
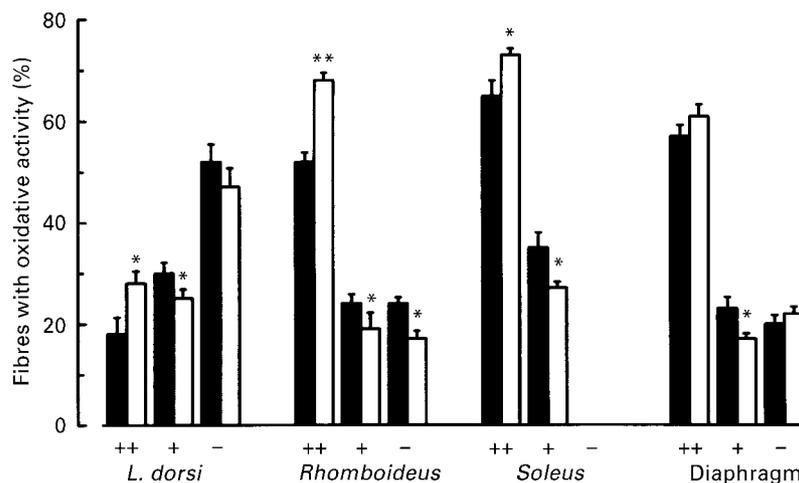

Fig. 6. Nutritional regulation of myofibre oxidative activity. Proportion of fibres with high (++), intermediate (+) or low (−) succinate dehydrogenase activity, in muscles from pigs on a high ((■), 6 % (60 g food/kg body weight per d)) or low ((□), 2 % (20 g food/kg body weight per d)) food intake. Mean values were significantly different from that of the high-intake group, * $P < 0.05$, ** $P < 0.01$. Values are means for six littermate pairs with standard errors of the means represented by vertical bars.

diaphragm respectively, were 7·0 (SEM 0·5), 34·0 (SEM 2·7), 43·0 (SEM 1·0) and 23·0 (SEM 2·8) for animals on the high intake, and 7·0 (SEM 1·3), 44·0 (SEM 0·8), 43·0 (SEM 2·2) and 22·0 (SEM 1·3) for animals on the low intake.

Comparison between type I fibre proportions estimated either by MyHC immunocytochemistry or by mATPase histochemistry indicated that for *l. dorsi* the values were almost identical. However, in diaphragm there was a striking difference between the two methods: only half the type I fibres identified by immunocytochemistry were observed using mATPase histochemistry (Fig. 1), and in *rhomboideus* and *soleus* the mATPase method also tended to underestimate the proportion of type I slow-twitch fibres.

Impact of nutrition on metabolic properties of functionally diverse skeletal muscles

Energy intake was found to have significant muscle-specific effects on myofibre oxidative and glycolytic properties, as determined by succinate dehydrogenase and α-glycerophosphate dehydrogenase activities respectively. In all four muscles examined, animals on the low compared with the high food intake had a greater proportion of fibres with high oxidative activity (Fig. 6). The relative increase was dependent on muscle type: 55 % in *l. dorsi* ($P < 0.05$), 30 % in *rhomboideus* ($P < 0.01$), 21 % in *soleus* ($P < 0.05$), and only 7 % in diaphragm ($P > 0.05$).

In relation to glycolytic properties, level of energy intake affected only *rhomboideus* and diaphragm, and had no effect on *l. dorsi* and *soleus* (Fig. 7). In *rhomboideus*, the proportion of fibres with high glycolytic activity was 16 % lower in animals on the low compared with the high intake ($P < 0.01$). In diaphragm, by contrast, there were significantly more glycolytic fibres in the low compared with the high intake group ($P < 0.01$); the proportion of high activity fibres (++) was upregulated at the expense of low activity

fibres (+), while the number of fibres without glycolytic activity (−) did not change.

Discussion

The major finding of this study is that postnatal nutritional status has a striking muscle-specific influence on type I slow MyHC expression, at both the mRNA and protein levels. Moreover, early nutrition has also been found to exert marked differential effects on the metabolic properties of functionally distinct muscles. These findings have important implications for both the immediate and long-term development of human infants. Before consideration of the potential mechanisms underlying these findings and their significance to human health and nutrition, it is first essential to discuss the suitability of the methodology available for investigating muscle development.

Evaluation of methods for assessing postnatal muscle development

A range of techniques was used in the present study to assess MyHC mRNA and protein expression, and contractile (type I slow or type II fast) and metabolic (oxidative or glycolytic) properties of myofibres. *In situ* hybridization and immunocytochemistry for type I slow MyHC expression provided clear-cut repeatable results, as did the use of histochemical techniques for succinate dehydrogenase and α-glycerophosphate dehydrogenase activities in a range of functionally distinct muscles. By contrast, mATPase activity did not always provide clear-cut results. Although mATPase staining at acidic and alkaline pre-incubation pHs can resolve most fibre types in mature muscle, this is not the case postnatally. Thus, acidic pH produced a broad spectrum of mATPase staining; fibres that were type I according to mRNA and protein expression appeared to be type I, IIA,
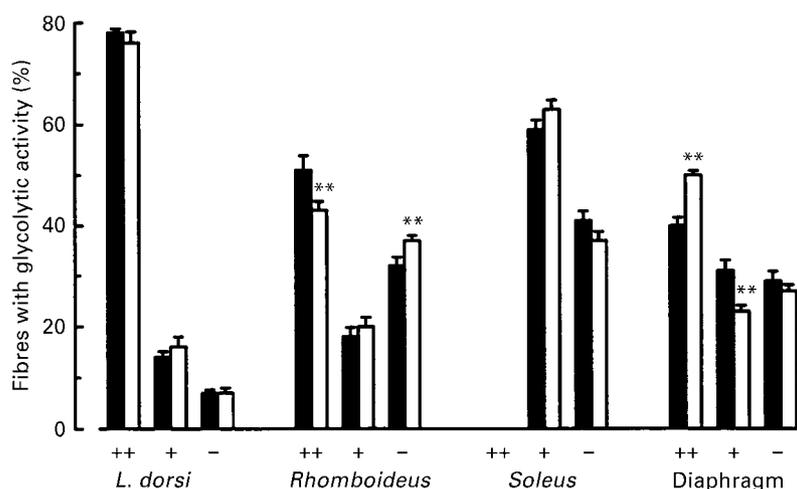

Fig. 7. Nutritional regulation of myofibre glycolytic activity. Proportion of fibres with high (++), intermediate (+) or low (−) α-glycerophosphate dehydrogenase activity, in muscles from pigs on a high ((■), 6 % (60 g food/kg body weight per d)) or low ((□), 2 % (20 g food/kg body weight per d)) food intake. Mean values were significantly different from that of the high-intake group, * $P < 0.05$, ** $P < 0.01$. Values are means for six littermate pairs with standard errors of the means represented by vertical bars.

IIB or IIC at this pH. Using an alkaline pre-incubation pH, however, it was possible to classify fibres as either type I or II, but even then a few type I fibres retained some mATPase activity, especially in diaphragm. It was known previously that during fetal and perinatal life, mATPase is resistant to both acidic and alkaline pH and many fibres appear to contain a mixture of slow and fast MyHC isoforms (Lefaucheur *et al.* 1995). Our results indicate that during postnatal development mATPase activity has not yet reached adult levels and therefore, even in species born at a relatively advanced stage of development, mATPase histochemistry should not be used to determine fibre type. Moreover, discrimination between type II subtypes at the protein level is not always possible because antibodies suitable for mature muscle cannot always distinguish between fibre types postnatally (Mascarello *et al.* 1992; Lefaucheur *et al.* 1995). It is clear, therefore, that any comprehensive assessment of postnatal muscle development must involve a range of techniques, including *in situ* hybridization, immunocytochemistry and enzyme histochemistry.

Nutritionally-induced changes in type I slow myosin heavy chain mRNA expression and muscle phenotype: implications for optimal development

The most striking change induced by nutrition was in type I slow MyHC mRNA expression. In *rhomboideus*, the proportion of fibres expressing this isoform was on average 53 % greater in animals on a low compared with a high energy intake. This difference was reflected in changes in *rhomboideus* phenotype: animals on the low intake had 18 % more fibres expressing type I MyHC protein, 30 % more fibres with type I mATPase activity, 30 % more oxidative fibres, and a decrease of 16 % in glycolytic fibres. The change in mRNA expression was greater than the differences in phenotype, suggesting that MyHC gene transcription is more sensitive to nutritional change than is translation. Precise mechanisms underlying this difference remain to be investigated. Energy intake also caused marked changes in metabolic activity and most striking was the finding that in *l. dorsi*, *rhomboideus* and *soleus* the proportion of myofibres with high oxidative activity was elevated on the low intake. This concurs with studies in *l. dorsi* from animals kept at 35°C or 10°C, in which a low intake resulted in a greater proportion of oxidative fibres (Dauncey & Ingram, 1988).

Overall, these results suggest that postnatal undernutrition can influence energetic efficiency via changes in myosin isoform gene expression and hence in myofibre phenotype. The relative increase in proportion of type I slow oxidative fibres on a low food intake will increase energetic efficiency when energy supplies are restricted because less ATP is required to generate the same amount of tension in slow-twitch than fast-twitch myofibres (Henriksson, 1990). Although this response will conserve energy, it may be detrimental to optimal muscle function. Hormone responsiveness and nutrient utilization are both dependent on myofibre type. The uncoupling proteins UCP2 and UCP3 have been implicated in regulation of lipids as fuel substrate and their responsiveness to food deprivation is muscle-type dependent (Samec *et al.* 1998). Moreover, suboptimal energy balance has recently been shown to selectively upregulate glucose transporter mRNA expression but reduce insulin-dependent glucose uptake in muscle during postnatal development (Katsumata *et al.* 1999). Thus, adverse effects may be widespread, involving changes in contractile and metabolic functions, insulin sensitivity and nutrient utilization. The effects may not only be immediate since alterations in nutrition occurring at critical stages of development can also have long-term or permanent effects (Widdowson & McCance, 1975; Lucas, 1994; Barker, 1995; Dauncey, 1997; Hoet & Hanson, 1999).

Mechanisms underlying regulation of type I slow myosin heavy chain mRNA expression and muscle phenotype

A number of mechanisms are implicated in the present findings. Two major regulators of muscle gene expression and myofibre differentiation are contractility and hormonal status. Moreover, in addition to energy balance, specific nutrients may be involved in the response. A key regulator should fulfil two important criteria: to be a specific regulator of muscle development and to be modulated by postnatal nutritional status. As discussed later, it is probable therefore that changes in hormones and their receptors played a pivotal role in our findings.

Muscle contractility has considerable potential for altering muscle development: mechanical loading and chronic low-frequency stimulation induce upregulation of the slower MyHC isoforms (Swoap *et al.* 1994), while mechanical unloading increases fast MyHC content (Goldspink *et al.* 1992). However, there were unlikely to be major differences in muscle contractility between the two treatment groups. For example, although the increased contractility associated with shivering could differentially affect myofibre types (Herpin & Lefaucheur, 1992; Harrison *et al.* 1996*a*), the animals were living within the zone of thermal neutrality. Second, specific nutrients may have been involved in the response to undernutrition. A restricted access to food does not only reduce energy intake but also modifies the availability of nutrients such as glucose, amino acids and vitamins which are known to regulate the expression of numerous genes, and may also regulate cell differentiation and maturation (Girard *et al.* 1994; Bruhat *et al.* 1999; Ferré, 1999). Although the precise role of specific nutrients in muscle development is unclear, their potential role at the molecular level is significant and probably involves direct actions at the gene level or via interactions with hormones (Dauncey & Gilmour, 1996).

Third, hormones may have acted as mediators of the nutritionally-induced changes in myofibre differentiation. As pointed out in the Introduction (pp. 185–186), many hormones including TH, growth hormone, insulin-like growth factor-I, insulin and glucocorticoids have been implicated in muscle development and these are all influenced by nutritional status. TH induce expression of MyHC isoforms with higher ATPase activity, in the sequence type I→IIA→IIX→IIB (d'Albis & Butler-Browne, 1993). Mild undernutrition reduces thyroid gland activity, plasma TH levels and muscle nuclear TH-receptor binding capacity (Dauncey & Gilmour, 1996; Harrison *et al.* 1996*b*; Morovat

& Dauncey, 1998) and these changes could thus increase type I slow MyHC, via an interaction of TH with nuclear receptors, which in turn activate or repress muscle-specific genes. Moreover, recent studies in our laboratory have highlighted muscle-specific differences in TH receptor isoform expression, which may account for differences in hormonal responsiveness between muscle types (White & Dauncey, 1999). It should be stressed that several hormones are probably involved in nutritional regulation of postnatal muscle development. For example, growth hormone appears to play a key role in increasing the oxidative capacity and proportion of type I slow fibres in muscle (Ayling *et al*. 1992), and a low food intake induces muscle-specific upregulation of growth hormone receptor mRNA expression that is closely related to myofibre type (Dauncey *et al*. 1994; Katsumata *et al*. 1997). Not only direct hormone action but also interactions between hormones are likely to be involved. For example, growth hormone receptor mRNA expression is modulated by TH and glucocorticoids (Duchamp *et al*. 1996; Li *et al*. 1996), highlighting the complexity of the regulatory system that probably functions *in vivo*. To summarize, it is probable that the present results were mediated by multiple factors and involved complex interactions between hormones acting as nutritional signals and differential effects on their cell membrane receptors or nuclear receptors.

Concluding remarks

This study has demonstrated that mild postnatal under-nutrition can critically influence muscle development at the molecular and functional levels. The precise mechanisms which regulate the differential effects of a low food intake on type I slow MyHC mRNA expression and myofibre phenotype now need to be investigated. Attention should focus on the role of TH receptor α and β isoforms (White & Dauncey, 1999), on interactions between hormones acting as nutritional signals, and on the direct actions of specific nutrients on muscle development. Such studies would not only elucidate the mechanisms by which nutrition regulates development but could also be used for reversing or ameliorating the adverse effects of early malnutrition.

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