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Two myogenic regulatory factor transcripts exhibit muscle-specific responses to disuse and passive stretch in adult rats

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Abstract Levels of myogenic regulatory factor (MRF) transcripts are altered in a muscle-specific manner in response to hind limb immobilisation of adult male rats, for a 2 day period, in either a lengthened or shortened position which result in passive stretch or disuse atrophy respectively. Myogenin transcript levels were dramatically elevated in the stretched plantaris but not soleus, whereas the MRF4 transcript was significantly elevated in soleus but not plantaris. Levels of myogenin mRNA were unaffected by disuse in either muscle and MRF4 was markedly lower in plantaris in response to disuse.

Key words: Stretch; Disuse; Immobilisation; Myogenin; MRF4

1. Introduction

Skeletal muscle is an extremely dynamic tissue capable of rapid qualitative and quantitative alterations in phenotype in response to a variety of external influences. This has been widely demonstrated with respect to the modulation of products of the myosin gene family. We have noted that muscle-specific responses in the expression of this gene family occur during both stretch-induced hypertrophy and disuse-induced atrophy produced by immobilising muscles at greater or less than resting length respectively [1].

Several studies suggest that these adaptive changes in skeletal muscle in response to environmental stimuli are the result of modulation at the transcriptional level [2] but the mediators involved in the transduction pathways between the external stimuli and altered muscle gene expression have yet to be identified. Skeletal muscle-specific proteins, known as the myogenic regulatory factors (MRFs), which bind to the regulatory regions of certain muscle genes and activate their transcription [3], have been extensively examined during mouse embryogenesis and skeletal muscle differentiation [4]. However, little is known about their role, if any, in adult muscle. One of these basic helix-loop-helix (bHLH) proteins, myogenin, which is crucial during development for complete muscle fibre formation, is also dramatically upregulated in adult muscle by denervation [5,6]. In contrast, MRF4, which is expressed later in muscle development than other MRFs and is present at higher levels than other bHLH proteins in adult muscle, shows a small or no elevation in response to denervation [5,6].

In this study we have employed an in vivo model, described previously [1], of muscle immobilisation in the shortened and lengthened position over a 2 day period. The levels of myogenin and MRF4 mRNAs were examined in these two situations in the fast phasic plantaris and slow postural soleus muscles.

2. Materials and methods

2.1. Animals and experimental protocol

Male Wistar rats were divided into two groups of six animals. In the first group one hind limb was immobilised under halothane anaesthesia using Vet cast glass fibre tape in a fully plantar-flexed position and in the second group the limb was immobilised in a dorsi-flexed position for 2 days after which the animals were killed. In the plantar-flexed limb the slow postural soleus and the fast phasic plantaris were immobilised at less than resting length whereas in the dorsi-flexed limb these muscles were immobilised at greater than resting length. The soleus and plantaris muscles were rapidly removed from the experimental and contralateral control limbs and immediately frozen in liquid nitrogen. During the experimental period the animals were closely observed and immediately removed from the study at the first sign of oedema.

2.2. RNA isolation and Northern analysis

Total cellular RNA was extracted from homogenised muscles using RNAzol-B (Biogenesis Ltd., Poole, UK) following the protocol described by the manufacturer (Cinna/BiotecX Bulletin No. 3). RNA samples (10 μg) were size separated on formaldehyde denaturing agarose (1%) gels. After removal of residual formaldehyde, RNA was transferred by capillary blotting onto nylon membranes (Hybond-N, Amersham International) and fixed to the membrane by baking at 80°C. cDNA fragments complementary to regions of the myogenin, MyoD and MRF4 transcripts were isolated after restriction digest of plasmids and were radioactively labelled by the random priming method using α-[^{32}P]dATP and a multiprime labelling kit (Amersham Int.) according to the manufacturer's instructions. Hybridisation of the cDNA probes to the membranes was carried out over 1 h at 68°C using Quickhyb solution and the associated procedure (Stratagene). Blots were washed 3 times at room temperature in 2×SSC, 0.1% SDS for 30 min and once at 60°C in 0.2×SSC, 0.1% SDS for 20 min and then exposed to Hyperfilm MP (Amersham Int.) at −80°C. Exposure times of each blot to X-ray films were optimised for both presentation and densitometric analysis. The densities of the bands on the Northern blots were measured using Seescan and related to μg of total RNA. Integrated peak areas/μg RNA isolated from immobilised muscles were expressed as a percentage of the value obtained for the contralateral control muscle.

2.3. Statistical analysis

The data are expressed as means and S.E.M. Statistical significance between experimental and contralateral control muscles was assayed by *t*-test and a *P* value of less than 0.05 was considered significant.

3. Results

Over this short time period of 2 days only small decreases in mass were observed in plantaris and soleus muscles (6–17%) when compared with contralateral controls following immobilisation in the shortened position. This loss was prevented by immobilisation in the lengthened position and in soleus muscles there was a slight increase in mass (13%) following this treatment.

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Figs. 1 and 3a show results of Northern analysis of total RNA isolated from plantaris muscles of rats whose hind limb had been immobilised in either the shortened or lengthened position and the contralateral control muscle. The examples shown in Figs. 1 and 2 were chosen following autoradiography in which the exposure times had been optimised to illustrate the differences between control and experimental muscles. Transcript levels were similar in muscles from all contralateral controls independent of immobilisation position in the experimental limb. In plantaris muscle when either MRF4 or myogenin cDNAs were used as probes no significant changes in the levels of either transcript were observed following immobilisation in the shortened position. Immobilisation in the lengthened position also had little effect on MRF4 transcripts but caused very dramatic increases in the levels of myogenin mRNA ($P < 0.001$).

Figs. 2 and 3b show that in soleus muscles immobilised in the shortened position there is little change in myogenin mRNA levels. In contrast, this treatment causes a significant fall in MRF4 levels ($P < 0.001$) over a 2 day period. Immobilisation in the lengthened position (passive stretch) over this period induced an increase in MRF4 mRNA levels but produced no significant change in myogenin levels in the soleus. These effects are distinctly different from those produced on MRF4 transcripts by disuse in soleus and stretch in plantaris muscle.

Northern analysis of total RNA from contralateral control muscles of animals whose limbs were immobilised in either the shortened or lengthened position showed similar, levels of either myogenin or MRF4 mRNAs (data not shown).

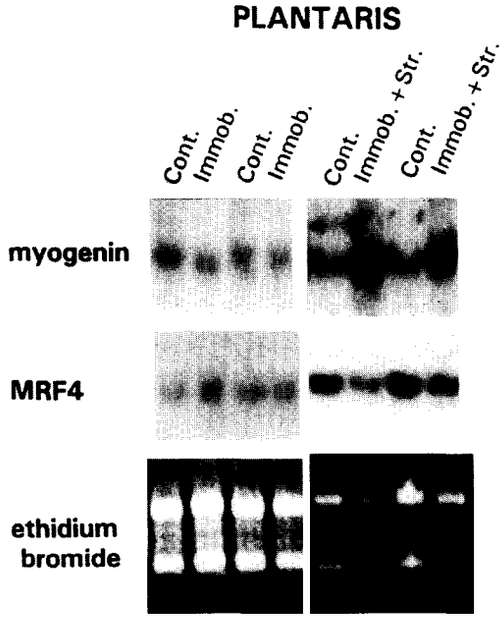

Fig. 1. Examples of Northern analysis of myogenin and MRF4 mRNAs in the plantaris muscle immobilised in either the shortened position (Immob.) or the lengthened position (Immob.+Str.). Ethidium bromide (E) staining of the blot was used to determine even loading of total RNA.

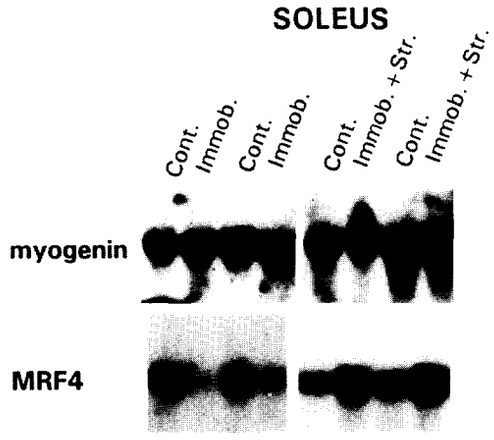

Fig. 2. Examples of Northern analysis of myogenin and MRF4 mRNAs in the soleus muscle immobilised in either the shortened position (Immob.) or the lengthened position (Immob. + Str.).

4. Discussion

The role of MRFs in the early development of skeletal muscle has been the subject of intense investigation for a number of years. However, several recent studies have indicated that the bHLH proteins may also play a role in the adaptive modulation of phenotype in adult muscle in response to a variety of stimuli [6,7]. The present study has extended these investigations by looking at the effect of mechanical tension on the transcript levels of two such proteins, myogenin and MRF4, in a fast and a slow skeletal muscle.

In the present studies on passive stretch the most dramatic response was the large elevation of myogenin mRNA levels in plantaris. This transcript was unaffected by passive stretch in soleus muscle where it is normally present at a higher level than in plantaris muscle. In contrast MRF4 mRNA levels were up-regulated by the anabolic stretch stimulus in the slow soleus muscle but not in fast plantaris muscle. It is of interest that in a recent study no alteration in transcript levels of either myogenin or MRF4 was observed in the fast tibialis anterior muscle in response to a much shorter 2 h stretch stimulus [8]. The levels of myogenin transcripts were unchanged in response to immobilisation in the shortened position in either muscle and MRF4 mRNA transcripts were down-regulated in the slow soleus but not in the fast plantaris following disuse. From these results it is clear that the responses in both myogenin and MRF4 mRNA levels to immobilisation in either position are muscle specific as previously observed for contractile protein [1] and metabolic enzyme [9] gene expression.

Previous work has shown that reduced neural activity produced by immobilisation in a shortened position induces disuse atrophy [10]. Elimination of neural input by denervation of muscle also produces rapid atrophy and in both situations changes in myosin isoform composition follow a similar pattern [11]. Despite these consistent changes in contractile properties in slow muscles undergoing atrophy due to either immobilisation or denervation the shifts in MRF mRNAs are distinctly different following each of these treatments. In all denervated muscles investigated so far, in both mammalian and avian systems, there is an up-regulation of myogenin of 100–200-fold [5,6]. In contrast, in the present studies the myogenin mRNA levels were unchanged in both muscles.

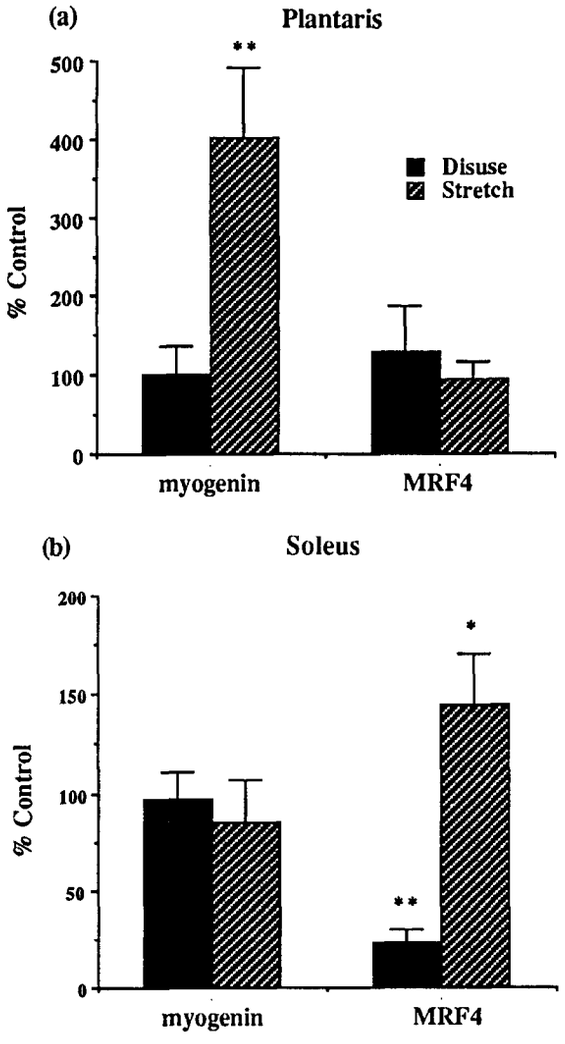

Fig. 3. Effects of disuse and stretch on myogenin and MRF4 mRNA levels in plantaris (a) and soleus (b) muscles from rats. The data are derived from absorbance values/10 μg of total RNA expressed as a percentage of the value obtained from the contralateral control muscle. Values are means ± S.E., $n = 4$–6, $^{**}P < 0.001$, $^{*}P < 0.02$.

In our study the most marked effect produced by disuse was the reduction of the MRF4 transcripts in slow postural soleus. This was surprising as MRF4 was less sensitive to modulation by denervation [5,6]. From these studies and the observations that MRF4 is expressed later in limb bud development [12] and is present at higher levels in postnatal muscle [5] than other MRF transcripts, particularly myogenin, it has been postulated that MRF4 has a crucial role in preserving the differentiated state rather than regulating muscle fibre phenotype. Our results suggest that there may be a more complex role for this particular MRF in modulating adult muscle gene expression.

In conclusion, both myogenin and MRF4 exhibited similar muscle specific responses, after disuse and passive stretch, to those we have previously observed in genes which contribute to the contractile properties of muscle. Patterns of expression of myogenin and MRF4 transcripts are distinctly different in disuse and denervation atrophy. Our study suggests that the regulation of adult muscle phenotype by MRFs may not be confined to MyoD and myogenin as previously suggested but MRF4 may also have a participatory role.

In the model we have employed disuse causes muscle fibre atrophy which is prevented by passive stretch and over longer time periods causes fibre hypertrophy [13]. Whether MRFs also play a role in the determination of this muscle fibre size is at present unclear. However, atrophy in fibres of transgenic animals has been observed where the inhibitory HLH protein Id, which forms heterodimers with MRFs and prevents DNA binding [14], is elevated, the degree of which relates directly to the level of expression of the transgene [15]. The potential of MRFs as regulatory factors in fibre size clearly requires further examination.

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