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Myogenin induces higher oxidative capacity in pre-existing mouse muscle fibres after somatic DNA transfer

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Muscle is a permanent tissue, and in the adult pronounced changes can occur in pre-existing fibres without the formation of new fibres. Thus, the mechanisms responsible for phenotype transformation in the adult might be distinct from mechanisms regulating muscle differentiation during muscle formation and growth. Myogenin is a muscle-specific, basic helix-loop-helix transcription factor that is important during early muscle differentiation. It is also expressed in the adult, where its role is unknown. In this study we have overexpressed myogenin in glycolytic fibres of normal adult mice by electroporation and single-cell intracellular injection of expression vectors. Myogenin had no effects on myosin heavy chain fibre type, but induced a considerable increase in succinate dehydrogenase and NADH dehydrogenase activity, with some type IIb fibres reaching the levels observed histochemically in normal type IIx and IIa fibres. mRNA levels for malate dehydrogenase were similarly altered. The size of the fibres overexpressing myogenin was reduced by 30–50 %. Thus, the transfected fibres acquired a phenotype reminiscent of the phenotype obtained by endurance training in man and other animals, with a higher oxidative capacity and smaller size. We conclude that myogenin can alter pre-existing glycolytic fibres in the intact adult animal.

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Fast muscle fibres have low levels of oxidative enzymes and rely on glycolysis for their energy supply. Glycolysis provides a rapid, but also rapidly depleted source of ATP, and fatigue develops after brief usage. It has been known since the 1970*s* that muscles subjected to endurance training adapt by increasing the level of oxidative enzymes, and thus improve endurance by shifting from a glycolytic to a more oxidative metabolism (Holloszy & Booth, 1976). This adaptation is dependent mainly on the pattern of electrical activity evoked in the sarcolemma by the motorneurons (Pette & Vrbová, 1992; Gundersen, 1998), and although the changes in molecular makeup can be quite profound, they occur mainly in pre-existing fibres without prior cell death and regeneration (Gorza *et al.* 1988; Pette & Vrbová, 1992; Gundersen, 1998).

During development, various transcription factors are known to regulate muscle gene expression. Among the most important are four muscle-specific basic helix-loop-helix proteins: MyoD, myogenin, MRF4 and Myf-5 (Weintraub, 1993; Molkentin & Olson, 1996). We have shown previously that when forced expression of myogenin is started during muscle development in transgenic mice, high levels of oxidative enzymes are induced (Hughes *et al.* 1999). Although this suggests that myogenin regulates oxidative capacity in developing fibres, the relevance for changes occurring in the adult, such as during exercise, remains unclear. In addition, the observed effects could have been indirect. The transgene was present in all cells and changes were triggered in a large number of fibres, leading to altered gross muscle performance, which in turn is likely to trigger changes in usage and nerve-evoked electrical activity. For example, if myogenin weakened the fibres as a primary effect, then an increased oxidative capacity might result from more frequent recruitment of high-threshold motor units.

Conventional transgenic animals are now readily available and have become popular even for problems related to adult animals, but we think it is also important to develop alternative strategies. In this paper we have used two different methods of somatic gene transfer: electroporation and single-cell injection. We show that when myogenin is overexpressed in identified fibres demonstrated to have existed before the intervention, these fibres were transformed into a more oxidative phenotype. Since only a few fibres were affected, reflex-mediated changes in the CNS-evoked activity pattern are unlikely. Our data suggest that myogenin has a cell-autonomous effect on oxidative properties in the permanent muscle fibres of adult animals.

METHODS

Animal experiments

The experiments were performed on female NMRI mice weighing 20–30 g. All surgical procedures were performed under deep anaesthesia after intraperitoneal injections of 5 μl g^{-1} Equithesin (Ullevål Apotek, Norway). The animal experiments were approved by the Norwegian Animal Research Authority and were conducted in accordance with the Norwegian Animal Welfare Act of December 20th 1974, no. 73, chapter VI, sections 20–22, and the Regulation on Animal Experimentation of January 15th 1996.

Plasmids

pAP-lacZ (Kisselev *et al.* 1995) contains the *Escherichia coli* β-galactosidase coding sequence driven by the Rouse sarcoma virus promoter. pCMS-lacZ was constructed from pAP-lacZ and pCMS-EGFP (Clontech, USA) by cutting the plasmids with *Hin*dIII and *Bam*HI and then ligating the 3.7 kb *Hin*dIII-*Bam*HI fragment directionally into the reduced pCMS-EGFP plasmid. This resulted in a plasmid expressing β-galactosidase driven by the cytomegalovirus (CMV) promoter. Rat myogenin was ligated into pCMS-lacZ in two steps. First a 1.5 kb *Eco*RI fragment containing the rat myogenin cDNA (Wright *et al.* 1989) was ligated into the EcoRI site of pCMS-EGFP. Cutting the resulting plasmid with *Hin*dIII made a new myogenin-containing fragment (3.1 kb). This fragment was ligated into the *Hin*dIII site of pCMS-lacZ. The resulting pCMS-lacZ-Mg plasmid expresses both the rat myogenin driven by the CMV promoter, and β-galactosidase driven by the simian virus 40 promoter. pM/Mg2 is an expression vector containing the rat myogenin cDNA driven by the myosin light chain 1 promoter and 1/3 enhancer, and pMDAF2 is essentially the same vector without the cDNA (for details see Gundersen *et al.* 1995). The plasmid pEGFP-N3 (Clontech) was used for expression of green fluorescent protein (GFP).

DNA electroporation

Transfection of DNA was performed essentially as described previously (Mathiesen, 1999). The extensor digitorum longus (EDL) muscle was exposed surgically, 10 μl of DNA (0.5–1 g l^{-1} in 0.9 % NaCl) was injected into the interstitium in the centre of the muscle from the distal end with a 701 Hamilton syringe. Subsequently, five trains of 1000 bipolar pulses (200 μs in each direction) with a peak-to-peak voltage of 20 V were run across the muscle by two 10 mm × 1 mm silver electrodes. Some electroporated muscles displayed fibres with central nuclei and other signs of regeneration. Fibres from such areas were excluded from further analysis.

Intracellular DNA injection

We have described previously the technique whereby single muscle fibres of intact mice are made transgenic by intracellular injection of DNA expression vectors through glass micropipettes with pressure pulses. Fluorescent dextran is co-injected with the DNA, and *in vivo* imaging techniques pioneered by Lichtman and colleagues (Lichtman *et al.* 1987; Balice-Gordon & Lichtman, 1990) allowed the identification of single cells during the injections, and of the same single cells when the muscle was re-exposed 2 weeks later. Before the muscles were frozen for histology, the injected fibres were examined under the microscope *in vivo*. *In vivo* imaging in this and a previous study (Utvik *et al.* 1999) revealed that cells that were damaged had a degenerated portion around the site of injection. The DNA remained localized to the injection site (Utvik *et al.* 1999), and such cells did not express reporter genes, and consequently did not contribute to our material.

Transfection in tissue culture

In order to test for expression of myogenin mRNA, human embryonic kidney cells (HEK-293) were transfected with pCMS-lacZ or pCMS-lacZ-Mg using the Lipofectamine2000 kit (Invitrogen). After 48 h, RNA was extracted from these cells and subjected to Northern analysis.

Histology and quantitative histochemistry

All experiments were performed on the EDL muscle, which comprises 73 % type IIb fibres, 18 % type IIx fibres, 9 % type IIa fibres and less than 0.1 % type 1 fibres (data not shown). The muscles were frozen slightly stretched in melting isopentane and stored at −70 °C before being cryosectioned at 10 μm. Serial sections were made in order to determine myosin heavy chain (MyHC) type and β-galactosidase, succinate dehydrogenase (SDH) and NADH dehydrogenase (NDH, EC 1.6.99.3) activity. MyHC type was determined with the following panel of monoclonal antibodies: BA-D5 (anti 1), SC-71 (anti IIa), BF-F3 (anti IIb) and BF-35 (anti all non-IIx; Schiaffino *et al.* 1989). β-galactosidase was visualized on the sections as described previously (Sanes *et al.* 1991), with modifications (Missias *et al.* 1996). Enzyme activity was visualized in reactions reducing tetrazolium to formazan, depending on the enzyme activity (Bancroft, 1975). Sections were also incubated without substrate, which resulted in uniformly weak or undetectable staining in all fibres. Bright-field pictures were taken through an Olympus compound microscope using a Hamamatsu C2400 CCD camera, and fluorescence images were taken with a Hamamatsu C2400 SIT camera. The cameras were connected to an ARGUS20 image-processing unit, and the digitized image was transferred to a Macintosh computer. Average pixel values and cross-sectional areas were measured in Photoshop (Adobe) by manually circling each fibre of interest. ARGUS20 determines 256 different grey levels for each pixel. To quantify histochemical staining, all measurements were performed in a darkened room, and measuring the light with no object in the light path standardized transillumination. All pixel values were within the linear range of the camera. Unless stated otherwise, the enzyme levels reported here were given relative to control muscle fibres on the same section. Thus, the mean pixel grey level of normal type IIb fibres in the same microscope field was set to 0 units, and the mean pixel grey level of normal type IIx fibres (the second most abundant fibre type in the EDL muscle) was normalized to100 units. Values from experimental fibres are reported using this relative scale.

mRNA quantification

RNA was isolated from frozen tissue by one-phase phenol extraction (Chomczynski & Sacchi, 1987) using TriReagent from the Molecular Research Center (OH, USA). mRNA was assayed and quantified either by Northern blotting or by quantitative competitive reverse transcriptase polymerase chain reaction (QCRT-PCR; Sun *et al.* 1996).

Northern blotting was performed essentially as described previously (Steensberg *et al.* 2002). The blots were either probed with nick-translated full-length rat cDNA (Fig. 2), or PCR-generated probes complementary to rat or mouse myogenin (Fig. 5). The following two primers were used: probe 1, Genbank M24393, 5′-CGG TGG AGG ATA TGT CTG TC-3′ and 5′-GGG CAC TCA CTG TCT CTC AA-3′; probe 2, Genbank D90156, 5′-CAC CCT GCT CAA CCC CAA C-3′ and 5′-CAG CCC CAC TTA AAA GCC C-3′. A control probe for 28S rRNA was generated by 5′-endlabelling of the oligonucleotide 5′-TCG CCG TTA CTG AGG GAA TCC TGG TTA GTT TCT TT-3′. Probes 1 and 2 were directed against rat and mouse sequences, respectively, but

hybridization was not species specific. Probe 1 corresponded to bases 659–1059 and Probe 2 to bases 355–795 in the rat sequence. For the QCRT-PCR assay, 1 μg total RNA was reverse transcribed into cDNA using the OmniScript Reverse Transcriptase (Qiagen, Hilden, Germany).

Plasmids containing cDNA fragments were generated by PCR on mouse muscle cDNA using the primers listed in Table 1 and inserted into the *Sma*I site of pBlueScriptII SK(+)(Alting-Mees & Short, 1989). These plasmids were used as templates for inverse PCR and recircularization, as described by Xu & Gong (1999). The resulting 'competitor' plasmids contained PCR products with a 50–112 bp internal deletion ('del' primers in Table 1).

For each primer set (Table 1), the competitive PCR reactions were performed by preparing a mastermix containing everything but the cDNA and the competitors. This mastermix was then divided out into four tubes containing the cDNA and different concentrations of the competitors. Each final PCR tube, a total of 15 μl, contained 5 μl diluted cDNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, dNTP (200 μM each), 10 μg μl^{-1} Ficoll 400, 1 mM tartrazine, 200 nM sense primer, 200 nM antisense primer (5′HEX labelled), 0.75 U Platinum *Taq* polymerase (Life Technologies, USA), and 5 μl competitormix. The competitormix consisted of equal amounts of a specific number of the five cut competitor plasmids diluted in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 1 ng ml^{-1} salmon sperm DNA (Sigma). The PCR reactions were performed on a PTC-200 (MJ Research, USA) with the following parameters: calculated mode; 94 °C, 5 min→(94 °C, 5 s→annealing temperature (T_a), 30 s→72 °C, 1 min) × 45→72 °C, 15 min→4 °C (T_a = 55 °C for glycerol-3-phosphate dehydrogenase (NAD^+) (GAPDH, EC 1.1.1.8), β-actin and myogenin; T_a = 60 °C for malate dehydrogenase (MDH, EC 1.1.1.37) and S26). After PCR, the two PCR products were separated on an alkaline (50 mM NaOH, 1 mM EDTA) agarose gel (2 % NuSieve 3:1 agarose, FMC BioProducts, USA). The bands were quantified with a fluorescence laser scanner (532 nm ImagerFX, Bio-Rad, USA). The point of equivalence (ratio = 1) was found by linear regression of the logarithm of the number of competitor molecules against the logarithm of the ratio between the two PCR products (Fig. 1*B*). Since the number of competitor molecules is known, this provides the number of target cDNA molecules.

To verify equal amplification efficiency between the wild-type and the competitor PCR products, known mixtures of wild-type and competitor plasmids were amplified (Fig. 1*A*). If the amplification efficiency is equal, the ratio between the input plasmids should be conserved for the resulting PCR products; this was confirmed.

Although RT-PCR is a very sensitive method for analysing transcripts, countermeasures should be taken to assure proper quantification. Thus, during the 'exponential phase', small variations in the amplification efficiency cause large differences in the outcome. Furthermore, late in the PCR reaction, the efficiency will decrease due to the reduction in primers and nucleotides. Most methods, like real-time PCR, quantify the PCR products before the end of the exponential phase, but still assume that the amplification efficiency is equal in all samples. In contrast, the competitive PCR assay used here bypasses the dependence on amplification efficiency in both the exponential and the plateau phase by co-amplification of a highly similar target, the competitor. Since the two targets are very similar, any changes in amplification efficiency will be the same for both targets. By using the ratio rather than the absolute amount produced, the potential problems with both the exponential phase and the plateau phase of competitive PCR are circumvented and a more reliable quantification is achieved. For more details see O'Connell *et al.* (1998) and Zimmermann & Mannhalter (1996).

Table 1. Primers for competitive reverse transcriptase polymerase chain reaction (QCRT-PCR)

Name	Sequence 5′→3′
Mouse myogenin sense	CAC CCT GCT CAA CCC CAA C
Mouse myogenin anti	CAG CCC CAC TTA AAA GCC C
Mouse MDH sense	TCT CTC AGT GTA CCC CGA AG
Mouse MDH anti	GAC TCA ACA CCC TCC ATC AA
Mouse GAPDH sense	GAA GGA GAT GCT AAA TGG GC
Mouse GAPDH anti	ACT TAA TGG GAC TTG GGA CC
Mouse β-actin sense	TTG GGT ATG GAA TCC TGT GG
Mouse β-actin anti	GAA AGG GTG TAA AAC GCA GC
Mouse S26 sense	AAA GAA GAA ACA ACG GTC GC
Mouse S26 anti	GGG TAA TTT TCC TTC CGT CC
Mouse myogenin del1	AGC AAA TGA TCT CCT GGG TT
Mouse myogenin del2	TCA CGG TGG AGG ATA TGT CT
Mouse MDH del1	CAC GAG GGA GAA GAC AAA GC
Mouse MDH del2	ACT TCT CTA CGC CCT TGC TC
Mouse GAPDH del1	ACC GCA GTG AAC AAG GGG A
Mouse GAPDH del2	CCA ACG GAG ACA AGC GGA A
Mouse β-actin del1	ACA TCT GCT GGA AGG TGG AC
Mouse β-actin del2	TGC TTC TAG GCG GAC TGT TA
Mouse S26 del1	GTG AGC TGT GCC ATC CAT AG
Mouse S26 del2	CGC TTG CTT CAG ATA TGT CC

Primer sequences used in the QCRT-PCR assay (see Methods).

Statistics

Differences were tested with ANOVA (Fisher's PLSD) using the Statview 5 software (SAS publishing, USA). $P < 0.05$ was considered significant.

RESULTS

We first confirmed that myogenin mRNA of the right size was produced in kidney cells in culture where high transfection efficiency can be obtained and where endogenous myogenin does not interfere with the measurements (Fig. 2). The myogenin sequence hybridized to a band corresponding to about 1900 bases, which would be right for the 1486 bases cDNA and assuming a poly A tail of 400 bases.

Having confirmed expression *in vitro*, in a first series of *in vivo* experiments muscle cells were transfected by electroporation, with vectors expressing either myogenin and the reporter β-galactosidase (pCMS-lacZ-Mg), or as sham, expressing β-galactosidase only (pCMS-lacZ). Two weeks later, cross-sections from the muscles were checked for fibre types and stained for β-galactosidase and SDH (EC 1.3.99.1; Fig. 3*A*). The SDH levels of 42 type IIb myogenin fibres and 57 sham fibres from seven different muscles were compared with those of 137 type IIb fibres from the same microscope fields that did not stain for

β-galactosidase. In fibres overexpressing myogenin, the average SDH level was increased from 0 to 60 units (see Methods), and many fibres had levels as high as those found in the normal type IIx fibres, which by definition averaged 100 units. Some even had levels similar to those found in the normal type IIa fibres, averaging 124 units (Fig. 3*B*). There were no effects of expressing β-galactosidase only, as such fibres had an average SDH level of 1 unit (Fig. 3*B*). In absolute terms, the average pixel value of normal non-transfected fibres was (average ± S.E.M.) 190 ± 2, 181 ± 1 and 143 ± 1 in type IIa, IIx and IIb fibres, respectively. The type IIb fibres transfected with the myogenin expression vectors displayed an average pixel value of 166 ± 2, which was significantly higher than for normal type IIb fibres ($P < 0.0001$).

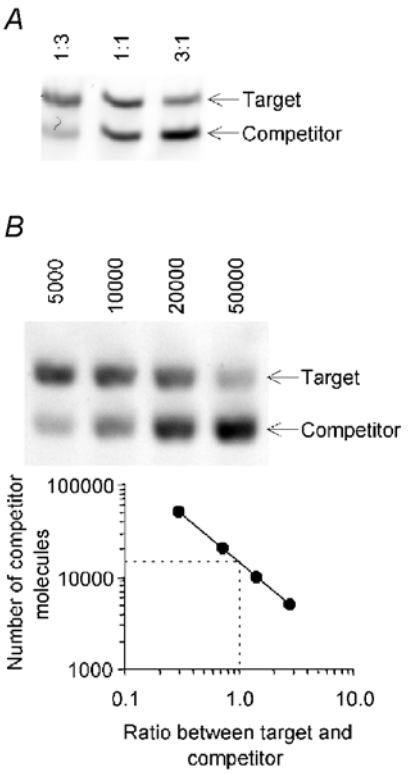

Figure 1. Examples demonstrating quantitative competitive reverse transcriptase polymerase chain reaction (QCRT-PCR) quantification

A, confirmation of equal amplification efficiency for wild-type and competitor templates. Mixtures (1:3, 1:1 and 3:1) of the two plasmids containing either myogenin wild-type or competitor PCR product were amplified by PCR and separated on a denaturing agarose gel. *B*, the top panel shows quantification of malate dehydrogenase (MDH) in a sample. Four different PCR reactions were performed spiked with different numbers of competitor plasmids (5000, 10 000, 20 000 and 50 000). The bottom panel in *B* shows the number of competitor molecules plotted against the ratio between the target and competitor band. By linear regression, the point of equivalence (ratio = 1) was found at 14 687 competitor molecules, that is, the sample contains 14 687 wild-type molecules.

Smaller fibre size aids rapid diffusion of oxygen from the surrounding capillaries into the fibre interior core, and in normal mice, oxidative fibres generally have lower cross-sectional areas than glycolytic fibres (Rivero *et al.* 1998, 1999). We found that forced expression of myogenin conferred a 39 % reduction in the cross-sectional area of type IIb fibres. Many fibres became as small as normal type IIx fibres, while expression of β-galactosidase after electroporation had no effect (Fig. 3*B*).

When SDH activity was plotted against fibre size there was no correlation between the two parameters for each fibre type. However, comparing the different IIa, IIx and IIb fibre types, a negative correlation between size and oxidative capacity was observed (Fig. 3*C*). Myogenin expression apparently had a concerted action on oxidative capacity and size such that this correlation was maintained, and the myogenin-expressing type IIb fibres formed a group between normal type IIb and IIx fibres (Fig. 3*C*).

Five type IIx fibres were also transfected and analysed. The material was too small to yield an accurate estimate of metabolic properties, but fibres were on average 28 % smaller than normal type IIx fibres ($P < 0.05$), and were in fact as small as normal type IIa fibres.

Electroporation frequently induces extensive tissue damage, which triggers regeneration, and might disturb, for example, innervation, circulation or interstitial components. A delayed expression has been reported, suggesting that the DNA is taken up and expressed in at least some regenerating fibres (Hoover & Kalhovde, 2000). On the basis of the electroporation experiments we could therefore not exclude the possibility that degeneration followed by regeneration was necessary for the changes we observed. Regeneration could trigger a developmental program that is not active in mature fibres, and thus our findings might not be relevant for understanding the mechanisms underlying changes in permanent fibres, such as that induced by exercise. To meet such objections, we injected DNA intracellularly into single fibres in the intact animal. This method (Utvik *et al.* 1999) causes minimal tissue damage and allows monitoring of the fate of single

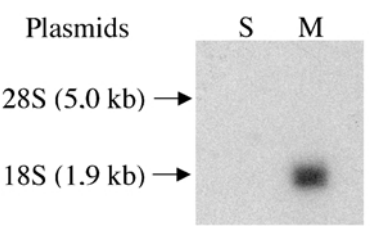

Figure 2. Myogenin mRNA production in HEK-293 cells

Northern blot of RNA extracts from human embryonic kidney (293) cells transfected with either the sham (S) plasmid pCMS-lacZ or the myogenin (M)-overexpressing plasmid pCMS-lacZ-Mg probed with rat myogenin sequence.

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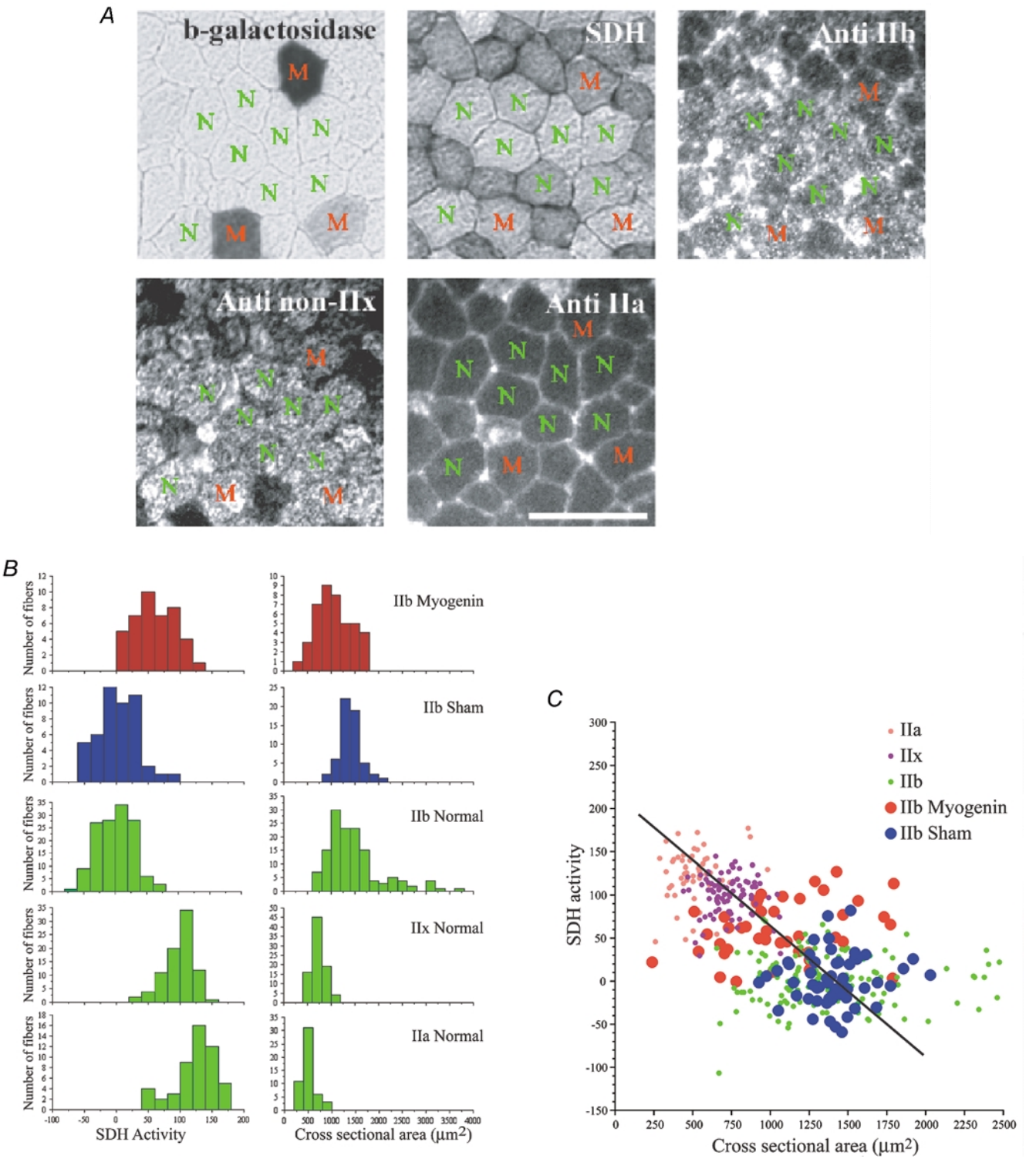

Figure 3. Effects on type IIb fibres of electroporation of a myogenin and β-galactosidase expression vector (pCMS-lacZ-Mg) or a sham vector (pCMS-lacZ) expressing β-galactosidase only into extensor digitorum longus (EDL) muscles

A, serial cross-sections stained for β-galactosidase, succinate dehydrogenase (SDH), or antibodies against myosin heavy chain (MyHC). The fibres marked M stained positively for β-galactosidase and were transfected with the vector overexpressing myogenin. The non-transfected normal type IIb fibres used as internal controls are marked with N. Scale bar = 75 μm. *B*, SDH levels and fibre cross-sectional area of type IIb fibres overexpressing myogenin (red), after transfection with sham DNA (blue), and of untransfected type IIb, IIx and IIa fibres within the same microscope fields (green). *C*, correlation between fibre cross-sectional area and SDH activity. Each dot represents one fibre. A few fibres with cross-sectional area > 2500 μm^2 were omitted from the scattergram in order to avoid compression of the data.

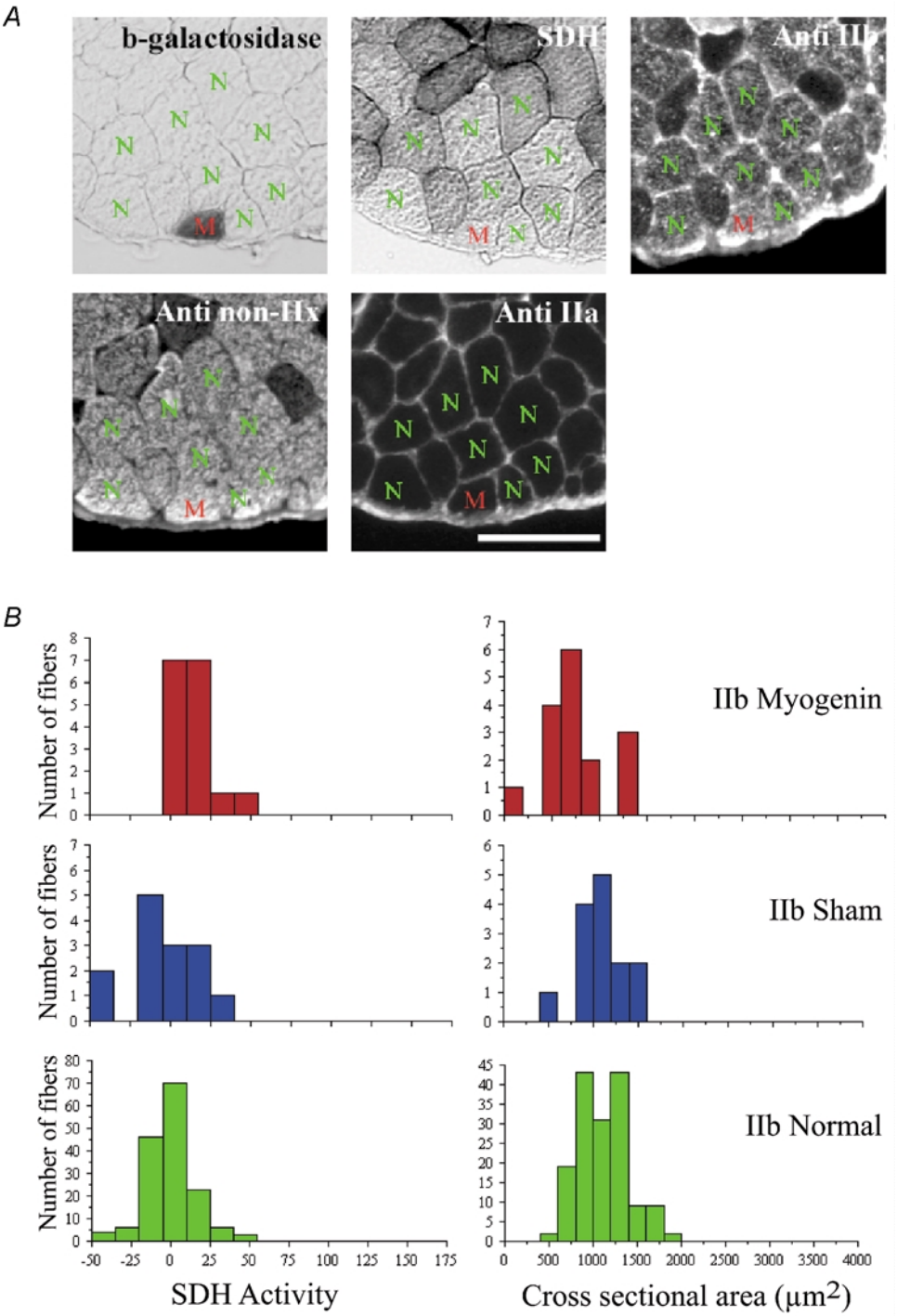

Figure 4. Effects of transfection by single-cell intracellular injection *in vivo* of a myogenin and β-galactosidase-expressing vector (pCMS-lacZ-Mg) or a sham vector expression β-galactosidase only (pCMS-lacZ) into type IIb fibres in the EDL muscle

A, serial cross-sections stained for β-galactosidase, SDH or antibodies against MyHC. The fibres marked M stained positively for β-galactosidase and were transfected with the vector overexpressing myogenin. Non-transfected normal type IIb fibre used as internal controls are marked with N. Scale bar = 75 μm. *B*, SDH levels and fibre cross-sectional area of type IIb fibres overexpressing myogenin (red) after injection of sham DNA (blue) and non-injected type IIb fibres within the same microscope fields (green).

Table 2. Oxidative enzyme activity in fibres transfected with plasmid mixtures

	Electroporation			Injection	
Enzyme	Myogenin	Sham	Control	Myogenin	Control
SDH	9.5 ± 3.6* (68)	0.5 ± 3.1 (46)	0.0 ± 1.2 (210)	10.2 ± 3.1* (8)	0.0 ± 1.2 (160)
NDH	26.6 ± 6.9* (41)	10.1 ± 5.2 (41)	0.0 ± 1.7 (169)	—	—

Effects on succinate dehydrogenase (SDH) and NADH dehydrogenase (NDH) activity in type IIb fibres measured histochemically after electroporation or single-cell injection of mixtures of pM/Mg2 and pAP-lacZ (myogenin) or pMDAF2 and pAP-lacZ (sham). Control fibres were randomly selected fibres of the same type within the same microscope field not staining for β-galactosidase. Enzyme activity is expressed relative to levels of non-transfected fibres in the same field (see Methods), and given as means ± S.E.M. The number of fibres is given in parentheses. * Significantly different from controls; Fisher's PLSD, $P < 0.05$.

cells by *in vivo* imaging. We analysed 29 β-galactosidase-positive type IIb fibres from 16 different muscles, and compared them with 160 non-injected type IIb fibres in the same microcope fields (Fig. 4*A*). After 2 weeks, the SDH activity in myogenin fibres increased to 14 units ($P < 0.05$), while sham injections had no effect (Fig. 4*B*). On average, the size was decreased by 34 % ($P < 0.05$). This effect on size was similar to the effect observed after electroporation, while the effect on SDH activity was considerably smaller. We speculate that this could be related to differences in the level of myogenin conferred by the different methods of transfection. Nonetheless, qualitatively the single cell experiments confirmed the results obtained by electroporation.

In a separate series of experiments we wanted to investigate whether the increased oxidative capacity was related to an increase in the mRNA levels for oxidative enzymes. To this end, we first electroporated muscles with a mixture of one plasmid expressing β-galactosidase (pAP-lacZ) and one plasmid either expressing myogenin (pM/Mg2) or a sham plasmid without expression (pMDAF2). The myogenin-expressing construct was different from that used above, but is the same as we have used previously for creating transgenic mice (Gundersen *et al.* 1995; Hughes *et al.* 1999). We first confirmed histochemically that this construct induced oxidative enzymes SDH and NDH (1.6.99.3; Table 2). The effects were smaller than in the previous series, but it is possible that the level of expression now conferred by the myosin light chain promoter/enhancer elements was lower than with the viral promoter used in the first series of experiments. Moreover, we have observed previously that when a mixture of plasmids is injected, 20 % of the expressing fibres only express one of the proteins (Utvik *et al.* 1999).

Having confirmed the effectiveness of this second myogenin expression vector in inducing an oxidative phenotype, we went on to measure mRNA. The myogenin expression vector pM/Mg2 was mixed with a GFP expression vector (pEGFP-N3) and electroporated into the muscle in order to visualize the transfected fibres in the living tissue (Fig. 5*A*). Subsequently, GFP-rich bundles of fibres were dissected out and subjected to mRNA quantification. First we analysed myogenin mRNA on a Northern blot (Fig. 5*B*). Bundles from muscles transfected with the myogenin-expressing plasmid showed a twofold increase in the signal compared to bundles transfected with pEGFP-N3 only (sham). The figures are an underestimate of the transcript level in expressing fibres since, even in the bundles that were dissected out, many fibres did not express the transgene. The increase was solely due to the transgene message, since the endogenous message was unaltered (as determined by a specific QCRT-PCR assay; Fig. 5*C*). Both endogenous and transgenic mRNA had a size of about 1.7 kb. For the transgene this is shorter than for the pCMS-lacZ-Mg plasmid (Fig. 2), and suggests that aberrant splicing might have taken place, as we have described previously in transgenic mice (Gundersen *et al.* 1995), Most of the biological activity should, however, be retained, since probe 1 hybridized with an intact sequence below codon 208 (Fig. 5*B*), and myogenin with truncations up to codon 158 has been shown to retain most of its ability to transactivate muscle promoters (Schwarz *et al.* 1992).

Having confirmed elevated levels of myogenin, we went on to investigate mRNA levels of metabolic enzymes and β-actin. MDH mRNA was increased by about 60 % in such bundles, while levels of the glycolytic enzyme GAPDH and β-actin were not significantly changed (Fig. 5*C*). Thus, myogenin seems to affect oxidative enzymes at the pre-translational level.

DISCUSSION

We have shown that the levels of the oxidative enzymes SDH, NDH and MDH are elevated and the fibre cross-sectional area is reduced when myogenin is overexpressed in adult, pre-existing fibres. The data suggest that the effect is at the gene-regulatory level, but changes in mRNA stability cannot be excluded. The changes occurred without changes in the major MyHC fibre type. Endurance training in man and in other animals can lead to pronounced changes in metabolic properties without MyHC fibre-type conversion (Saltin & Gollnick, 1983;

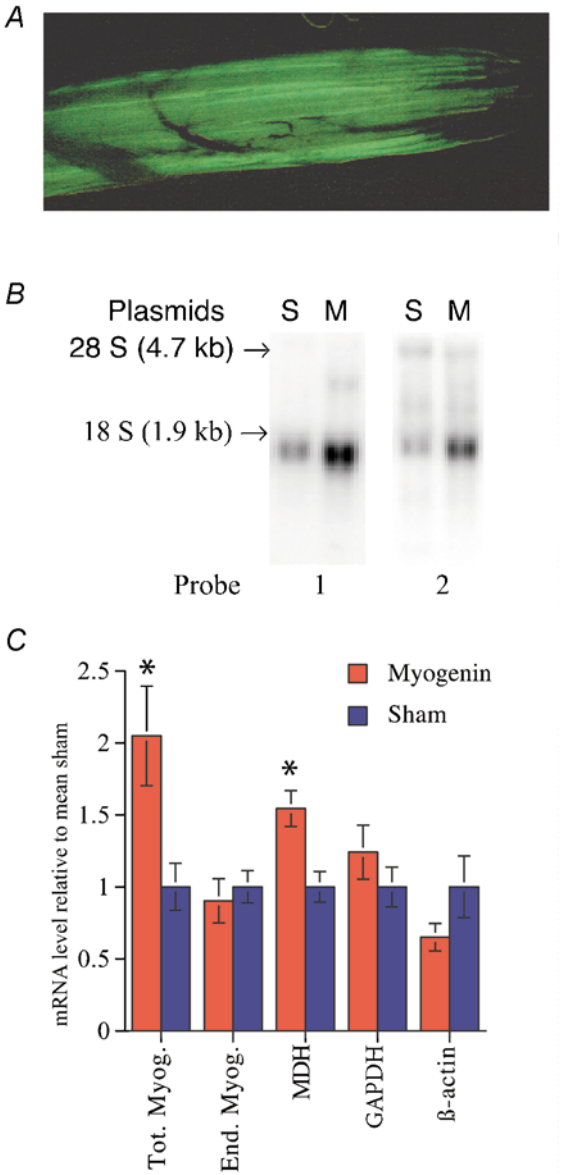

Figure 5. mRNA quantification after electroporation

A, EDL muscle illuminated with blue light after transfection with a mixture of green fluorescent protein (GFP) and myogenin expression vectors prior to dissection of GFP-rich fibre bundles for RNA analysis. *B*, Northern blots of myogenin mRNA from muscle fibre bundles electroporated with a myogenin + GFP-expressing plasmid (M, plasmid pEGFP-N3 and pM/Mg2) or a GFP-expressing plasmid plus a non-expressing plasmid (S, pEGFP-N3 and pMDAF2). The blot was probed with a sequence hybridizing against bases 659–1059 (probe 1) or 355–795 (probe 2) in the rat sequence. *C*, quantification of relative mRNA levels for total myogenin (transgenic plus endogenous), and mRNAs produced by the endogenous gene for myogenin, MDH, GAPDH and β-actin measured in fibre bundles that had strong GFP expression 12 days after electroporation with pEGFP-N3 and pM/Mg2 (myogenin, red) or pEGFP-N3 and pMDAF2 (sham, blue). Each column shows the mean ± S.E.M. of 6–10 muscles relative to the mean of the sham muscles. * Statistically significant difference between myogenin and sham (Fisher's PLSD, $P < 0.05$). Total myogenin was measured by Northern blotting, the other mRNAs by a QCRT-PCR assay (see Methods). Tot. Myog. = total myogenin; End. Myog. = endogenous myogenin.

Henriksson & Hickner, 1994; Fitts & Widrick, 1996), although changes in MyHC type within type II (e.g. from type IIb/IIx to IIa) can also occur (Abdelmalik *et al.* 1993; Wang *et al.* 1993; Kraemer *et al.* 1995). Hybrid fibres with more than one MyHC were occasionally observed in our muscles, but no more frequently in experimental fibres than in normal fibres, and such fibres were excluded from our material (< 1 %). MyHC is a very stable protein, and with the short observation periods used in the present study, changes in MyHC gene expression cannot be excluded. However, MyHC was also unaltered in transgenic mice where myogenin was overexpressed throughout development from the time of muscle formation (Hughes *et al.* 1999). No dose–response curve was available, so it cannot be excluded that higher doses would have influenced MyHC as well, but even if MyHC type were not regulated by myogenin, it is likely that other aspects of muscle speed were influenced. Thus, forced expression of myogenin in L6 cells in culture facilitated suppression of the 'fast' isoform of the sarcoplasmic Ca^{2+}-ATPase (SERCA1; Thelen *et al.* 1998), and we speculate that twitch duration might be altered by myogenin *in vivo*.

Oxidative fibres are smaller than glycolytic fibres, and it is believed that this facilitates oxygen transport into the fibre core. Our results suggest that oxidative capacity and size are regulated by similar mechanisms, or that size is decreased as a consequence of increased oxygen demand from the fibre. Animal studies on endurance training (Tamaki, 1987; Essen-Gustavsson *et al.* 1989) and endurance-training-like electrical stimulation (Pette & Vrbová, 1992) have similarly revealed a decrease in fibre cross-sectional area. In studies of endurance training in humans, the results have been more varied depending on the nature and duration of the training (Salmons & Henriksson, 1981). During such exercise, muscle fibres are subjected to complex changes in electrical activity and load, which are both potent in influencing fibre size. Variability in such parameters might explain the differences in results obtained by exercise. In the present experimental models such factors are reasonably constant, and they might be more suitable for unravelling the underlying mechanisms.

The present data suggest a causal relationship between myogenin and oxidative capacity in muscle, which is also supported by correlations found *in vivo*. For example, myogenin is more highly expressed in fibres with high oxidative capacity and mitochondrial content than in glycolytic fibres, both in rodents and fish (Hughes *et al.* 1993; Voytik *et al.* 1993; Rescan *et al.* 1995). Moreover, changes in the level of myogenin parallel fibre-type conversion in cross-innervation experiments, and after electrical stimulation and manipulating thyroid hormones (Hughes *et al.* 1993; Putman *et al.* 2000). Evidence indicates that myogenin acts in concert with the other

myogenic factors in the regulation of fibre phenotype, and it has been suggested that the myogenin/MyoD ratio rather than absolute levels might be decisive (Hughes *et al.* 1993). Several lines of evidence support this 'yin/yang' hypothesis: (1) denervation that leads to a strong upregulation of all of the myogenic factors (Eftimie *et al.* 1991; Carlsen & Gundersen, 2000) has no significant effect on metabolic enzymes (Gundersen *et al.* 1988); (2) forced expression of myogenin in transgenic mice, which is accompanied by a reduction in MyoD and MRF4 (Gundersen *et al.* 1995), has strong effects on metabolic enzymes (Hughes *et al.* 1999); (3) transgenic mice lacking functional MyoD have shifts in fibre type in the slow/oxidative direction (Hughes *et al.* 1997; Seward *et al.* 2001); (4) transformation in the slow direction by a combination of hypothyroidism and electrical stimulation is accompanied by a selective increase in myogenin with unaltered myoD levels (Putman *et al.* 2000).

Recent evidence suggests that MRF4 is also involved the regulation of muscle phenotype, since it is found predominantly in slow/oxidative fibres in some muscles (Walters *et al.* 2000). In addition, myogenic factors have been shown to interact closely with the MADS domain MEF2 proteins (Molkentin *et al.* 1995), which have also been implicated in fibre-type-specific gene regulation (Wu *et al.* 2000).

Several pathways might work upstream of the myogenic factors. High amounts of low-frequency activity are believed to be the most potent signal changing adult fibres in the slow/oxidative direction (Gundersen, 1998). It has been suggested that this leads to a sustained low-level increase in the steady-state $[Ca^{2+}]_i$, which in turn activates Ca^{2+}–calmodulin-activated phosphatase and calcineurin. Although there have been negative reports (Dunn *et al.* 2000; Swoap *et al.* 2000), there is mounting evidence suggesting that calcineurin is involved in the determination of the slow/oxidative phenotype in the adult (Chin *et al.* 1998; Dunn *et al.* 2001; Serrano *et al.* 2001). Interestingly, in cultured myotubes, a moderate increase in $[Ca^{2+}]_i$ increases the level of myogenin and not the other myogenic factors (Thelen *et al.* 1998). These effects could be coupled by calcineurin, since this factor can activate the myogenin gene (Friday *et al.* 2000). Thus, our findings would be consistent with the idea that myogenin might work downstream of calcineurin in inducing an oxidative phenotype in muscles subjected to high amounts of activity. In addition to electrical activity, a potent signal for muscle change is thyroid hormones. In hyperthyroid animals, the muscle fibre type changes in the fast direction. Myogenin might modulate or counteract some of the hormonal effects, as suggested by the decreased ability of T3 to induce the fast SERCA1 promoter when myogenin is overexpressed in L6 cells (Thelen *et al.* 1998).

In conclusion, our data suggest that myogenin is an important gene regulatory factor mediating changes in the 'slow' direction in pre-existing adult fibres. Since myogenin is regulated by electrical activity and can itself modulate hormonal effects, it might be involved in mediating both electrical and hormonal signals to the muscle cells. Such signals are crucial when muscles adapt to changing conditions.

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