The temporal and cellular expression of *c-fos* and *c-jun* in mechanically stimulated rabbit latissimus dorsi muscle

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The levels of *c-fos* and *c-jun* mRNA were measured by reverse transcription PCR in the rabbit latissimus dorsi muscle following three separate training regimes, i.e. passive stretch, 10 Hz electrical stimulation or a combination of the two. Both *c-fos* and *c-jun* mRNA expression peaked at around 1 h after imposing stretch and at around 4.5–6 h after the initiation of electrical stimulation. The combined stretch/electrical stimulation regime induced biphasic expression of both *c-fos* and *c-jun* mRNA, with peaks coinciding temporally with those for the individual regimes. Immunostaining with anti-Fos and anti-Jun antibodies revealed the accumulation of these proteins in both myofibre and interstitial cell nuclei following passive stretch. In contrast, following electrical stimulation the localization of immunoreactive c-Fos and c-Jun proteins was predominantly in interstitial cell nuclei. c-Fos and c-Jun immunoreactivity was also clearly co-localized in a proportion of myonuclei from stretched muscle. These findings suggest that the rapid induction of *c-fos* and *c-jun* is an early event in response to mechanical stretch and might trigger [via activator protein-1 (AP-1) transcriptional factors] events leading to muscle fibre hypertrophy. However, the involvement of AP-1 in inducing the phenotypic changes in muscle fibres as a result of electrical stimulation appears less clear.

INTRODUCTION

Cellular oncogene expression is down-regulated in post-mitotic striated muscle as muscle-specific genes are induced during muscle differentiation [1]. Expression of *c-myc*, *c-fos*, *c-jun* and other early response genes can be re-induced, in both cells in culture and in intact tissues *in vivo*, in response to a range of diverse stimuli [2,3]. In intact striated muscle, a variety of mechanical stimuli have been shown to lead to a rapid (within a few hours) and transient increase in the expression of a number of cellular oncogenes. These include pressure overloading of the heart [4], increasing coronary perfusion pressure [5], tenotomy of a functional synergistic muscle or administration of β-agonists [6]. Since all of the above cause hypertrophy of the muscle it is possible that changes in cellular oncogene expression are early events in the signal cascade which leads to muscle hypertrophy.

The c-Fos and c-Jun proteins are components of the activator protein-1 (AP-1) transcription factor complex [7,8]. Other members of the Fos and Jun gene families (e.g. Fos-B, Fra-1, Jun-B, Jun-D) also form complexes which bind to AP-1 binding sites. The mammalian AP-1 transcription factor comprises Fos–Jun heterodimers or Jun–Jun homo- or hetero-dimers, forming a variety of transcriptional regulators ([9] and refs. therein). The cellular role of these proteins in many cells is in transcriptional regulation as an adaptive response to external stimuli transduced via second messengers [10]. However, such a role has not been proven to occur in striated muscle tissue. In order to investigate further the roles of *fos* and *jun* cellular oncogenes in muscle cell signalling we have studied their expression under conditions known to induce either hypertrophy or alterations in gene expression.

We have previously demonstrated that simultaneous stretching and electrical stimulation (10 Hz) of skeletal muscle leads to both transformation from glycolytic fast-twitch to oxidative slow-twitch fibres and an increased rate of protein synthesis, leading to muscle hypertrophy [11,12]. Either electrical stimulation [13,14] or stretch alone [11,15] is capable of reprogramming gene expression to bring about changes in muscle fibre types. Electrical stimulation alone, however, causes substantial muscle atrophy (up to 45% after 3 weeks) [16,17]. Combining stretch with electrical stimulation speeds up the muscle transformation process significantly and produces muscle growth rather than atrophy [11,12]. The anabolic effects of the combined mechanical regime can be attributed to the stretch component [18].

In the present study we have employed reverse transcription (RT)-PCR to investigate the temporal expression of *c-fos* and *c-jun* mRNA in rabbit latissimus dorsi (LD), a predominantly fast-twitch muscle [19], in response to three mechanical training regimes (i.e. stretch alone, electrical stimulation alone or a combination of both) to determine which induces the earliest detectable changes. In addition, the cellular site of the increased expression has been investigated using immunolabelling techniques to determine the type and distribution of cells expressing c-Fos and c-Jun as a result of either stretch alone or electrical stimulation.

MATERIALS AND METHODS

Chemicals and DNA standards

The *c-fos* plasmid, comprising a 2.2 kbp rat cDNA insert cloned into the *Hin*dIII-*Bam*HI site of pCMV, was donated by Matthew Hope (University of Leeds). A 1.0 kbp *v-jun* sequence cloned into the *Bam*HI-*Eco*RI site of pSPT19 was used as the *c-jun* standard. Slow myosin primers were supplied by Kay Gillott (University of Leeds). Taq DNA polymerase was purchased from Boehringer Mannheim UK and AMV reverse transcriptase from Promega. Sheep polyclonal antibodies against c-Fos and c-Jun were purchased from Cambridge Research Biochemicals (OA11-824

Abbreviations used: RT, reverse transcription; AP-1, activator protein-1; LD, latissimus dorsi; DAB, 3,3′-diaminobenzidine.

and OA11-837 respectively). Chemicals were either AnalaR or molecular biology grade.

Operative procedures and experimental regimes

Dwarf Dutch rabbits, weighing 1–1.2 kg, were used throughout. Rabbits were maintained under Halothane anaesthesia and full aseptic procedures were employed for all operations. The rabbit LD muscles were subjected to acute (0.25–24 h) stretch, electrical stimulation or a combination of the two training regimes.

Passive stretch was imposed on the LD by the insertion of a silastic tissue expander between the LD and the ribs through an incision close to the spine and approximately half way along the muscle's length. The insertion was closed around the tubing of the expander and the filler port placed subcutaneously on the animal's flank. Each tissue expander was filled with 40 ml of sterile saline injected via the filler ports. This form of static stretch extends the muscle approximately 15 % beyond its resting length.

Electrical stimulation of the LD was achieved by the implantation of stainless-steel electrodes below the LD across the thoracodorsal nerve. Electrodes were attached to an external stimulator which was carried on the rabbit's back in a jacket. The stimulators delivered impulses of 3–5 V at a frequency of 10 ± 0.5 Hz with a pulse width of 1 ms.

Both procedures were carried out on animals undergoing stretch and stimulation of the LD. Animals were allowed to recover from the surgery for 24 h before the treatment protocols commenced.

After the appropriate time period (i.e. between 0 and 24 h), animals were humanely killed with an overdose of Sagatal (pentobarbitone) administered via an ear vein. The skin on the back was immediately opened and where muscles were taken for RNA analysis the rabbit was plunged into ice-cold saline to chill the LD. Both experimental and contralateral (i.e. internal control) LD muscles were quickly dissected out and portions of muscle from the central region were immediately frozen in liquid nitrogen and stored at −80 °C. For immunocytochemical analysis LD muscles were dissected dry. Small pieces from the centre of the muscle were mounted between blocks of liver, embedded in Tissue-Tek and dusted with fine talc before being flash frozen in liquid nitrogen.

Additional control muscles were obtained from non-operated and sham-operated animals. Sham stretch conditions were obtained by fitting an uninflated expander under the LD and sham electrical stimulation was achieved by implanting electrodes and fitting a stimulator which was never switched on.

RNA extraction and analysis by PCR

Samples (approx. 100 mg) of contralateral and experimental muscles were homogenized in 4 M guanidine isothiocyanate/25 mM sodium citrate (pH 7)/0.5 % sarkosyl/0.1 M 2-mercaptoethanol (1 ml/100 mg muscle) using a Polytron PT 10-35. Total RNA was extracted from the muscle samples according to the method of Chomczynski and Saachi [20]. A 2 μg aliquot of total RNA (measured spectrophotometrically at 260 nm) from each sample was subsequently reverse transcribed to yield cDNA as follows: RNA was incubated at 65 °C for 10 min with 0.2 μg of oligo $(dT)_{15}$ primer and then snap-cooled on ice before incubation at 42 °C for 1 h with AMV reverse transcriptase, RNasin (ribonuclease inhibitor) and dNTPs. PCR was carried out on a Techne PH-C thermal cycler. *c-fos* primers were designed (from a consensus sequence of rat, mouse and human *c-fos*) to yield a 261 bp product spanning intron 2 so that genomic contamination could be detected. The sequences of the forward and reverse *c-fos* primers were 5′-ACCAGCCCAGACCTGCAGTGG-3′ and 5′-CCGGCACTTGGCTGCAGCCAT-3′ respectively; these corresponded to 63–84 bp downstream of intron 1 (forward primer) and 57–36 bp upstream of intron 3 (reverse primer) on the *c-fos* gene [21]. *c-jun* primers yielded a 267 bp product corresponding to nucleotide numbers 1696–1963 on the *c-jun* gene [22]. The *c-jun* primer sequences were: 5′-CCCCTGTCCCCCATCGACATG-3′ (forward) and 5′-TTGCAACTGCTGCGTTAGCAT-3′ (reverse). PCR conditions were as follows: 28 (*c-fos*) or 24 (*c-jun*) cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, preceded by a denaturation step of 95 °C for 5 min and followed by an elongation step of 72 °C for 10 min. DNA standards were amplified alongside the cDNA samples: serial dilutions of rat *c-fos* or *v-jun* plasmids were used to provide semi-quantitative analysis of the samples. All cDNA samples and standards were amplified in duplicate. PCR using rabbit slow myosin heavy chain primers was carried out under previously determined conditions (see [19]).

Competitive PCR was carried out using 'mimic' *c-fos* and *c-jun* competitors [23]. These were created using composite primers which had sequences complementary to a stretch of pBR322 and also to *c-fos* or *c-jun*. PCR with the composite primers yielded a product which had the *c-fos* or *c-jun* sequences spanning part of the pBR322 sequence and was of a length designed to be distinguishable from the target cDNA products on an agarose gel, i.e. 218 and 206 bp for *c-fos* and *c-jun* mimics respectively, compared with 261 and 267 bp for the authentic *fos* and *jun*.

c-fos and *c-jun* PCR products were sequenced using Promega 'fmol' sequencing kit according to the manufacturer's instructions.

PCR products were run on a 2 % agarose/ethidium bromide gel and the negative of a photograph of the gel was scanned using an LKB laser densitometer. Reaction parameters were predetermined to ensure that amplification was within the exponential phase and that the absorbance values measured by the densitometer were within the linear range of the film.

Immunocytochemistry

The polyclonal antibodies used in this study were raised against short synthetic peptide sequences selected from conserved regions of mouse and human c-Fos or c-Jun (thus enhancing antibody specificity). Localization of c-Fos and c-Jun immunoreactivity was carried out using the peroxidase-labelled biotin/avidin system (Vectastain Elite ABC kit, Vector Laboratories Ltd.). Muscle was sectioned to 7 μm at −25 °C (serial sections cut to ≈ 5 μm), thaw-mounted onto gelatine-dichromate coated slides and air dried at room temperature for 1 h. Slides were subsequently stored at −80 °C. All incubations were carried out at room temperature unless indicated otherwise. Sections were fixed in ice-cold acetone for 30 min and endogenous peroxidase activity removed by incubation in methanolic H_2O_2 (1 %) for a further 30 min. After rinsing in PBS (pH 7.4) the sections were non-specifically blocked with normal horse serum (10 %) for 30 min. The sections were then incubated overnight at 4 °C in a humidified atmosphere with polyclonal immunoglobulins (IgG) directed against c-Fos or c-Jun at respective dilutions of 1:250 and 1:2000 in 10 % horse serum in PBS. Following three washing steps in PBS for 10 min each, the sections were incubated for 1 h with biotinylated rabbit anti-(goat IgG) (Dako Ltd., U.K.) diluted 1:400 in 10 % horse serum in PBS. After rinsing, the secondary antibody was revealed using the Vectastain ABC-HRP kit. Peroxidase activity was visualized with 3,3′-diaminobenzidine (DAB) (0.04 %) and H_2O_2 (0.01 %) and enhanced with nickel chloride (0.05 %). Sections were then lightly stained with

eosin, dehydrated in ethanol, cleared into xylene and mounted before being photographed.

Immunocytochemical controls to the experimental sections were incubated as above, either replacing the anti-Fos or anti-Jun antibodies with non-immune sheep serum, or omitting the primary antibodies altogether. In addition, antibody specificity was ascertained by pre-absorption of both polyclonals with their respective peptide antigens (Cambridge Research Biochemicals) and by immunoblot analysis.

Expression of results

c-fos and *c-jun* mRNA levels for experimental and contralateral muscles are expressed as fold increases over external control muscle values. Absorbance values (from experimental, contralateral and external control samples) were converted into picograms of DNA using the standard curves and fold increases were calculated from these numbers.

RESULTS

PCR

PCR amplification of the muscle cDNA samples with the *c-fos* or *c-jun* primers gave single products when run on an agarose/ethidium bromide gel, demonstrating that no genomic contamination was present in the total RNA preparations (Figure 1a). The sizes of the products (261 and 267 bp) were judged to be correct when compared with a DNA bp ladder, and the PCR products were shown to be those of *c-fos* or *c-jun* by sequencing around 180 bp of each. Figures 1(b) and 1(c) shows a typical standard curve obtained following PCR of the *c-fos* cDNA serial dilution with *c-fos* primers.

The relative efficiency (between samples) of the RT step was checked on selected batches of cDNA by carrying out PCR using primers to slow myosin heavy chain, since the expression of myosin does not change in response to the experimental protocols within the short time periods studied here (K. L. Gillott, V. M. Cox and D. F. Goldspink, unpublished work). Fold increases (over control values) of slow myosin mRNA in four randomly selected batches of experimental samples ranged form 0.7 to 1.4, demonstrating that myosin expression was uniform between cDNA samples from a given batch. It was therefore concluded that the RT step was consistent and reproducible between samples and that routine myosin analysis for every cDNA sample was unnecessary.

Results of the time-course of the expression of *c-fos* and *c-jun* mRNA in response to either stretch, electrical stimulation or a combination of stretch and stimulation are shown in Figures 2, 3 and 4. Sham-operated muscle samples showed baseline *c-fos* and *c-jun* expression and in all cases values were not significantly different from control (unoperated) values. Both *c-fos* and *c-jun* showed peak expression at 1 h in LD muscles subjected to stretch alone (Figures 2a and 2b). Whereas *c-fos* expression gave a discrete peak at 1 h, *c-jun* expression was apparently more complex and a secondary rise in expression was also seen at around 4.5 h of stretch. Small, insignificant changes (relative to external controls) were consistently found at all time-points in the internal control (contralateral) LD muscles. The electrical stimulation regime caused a later response, when compared with stretch, in that the expression of both genes was highest after

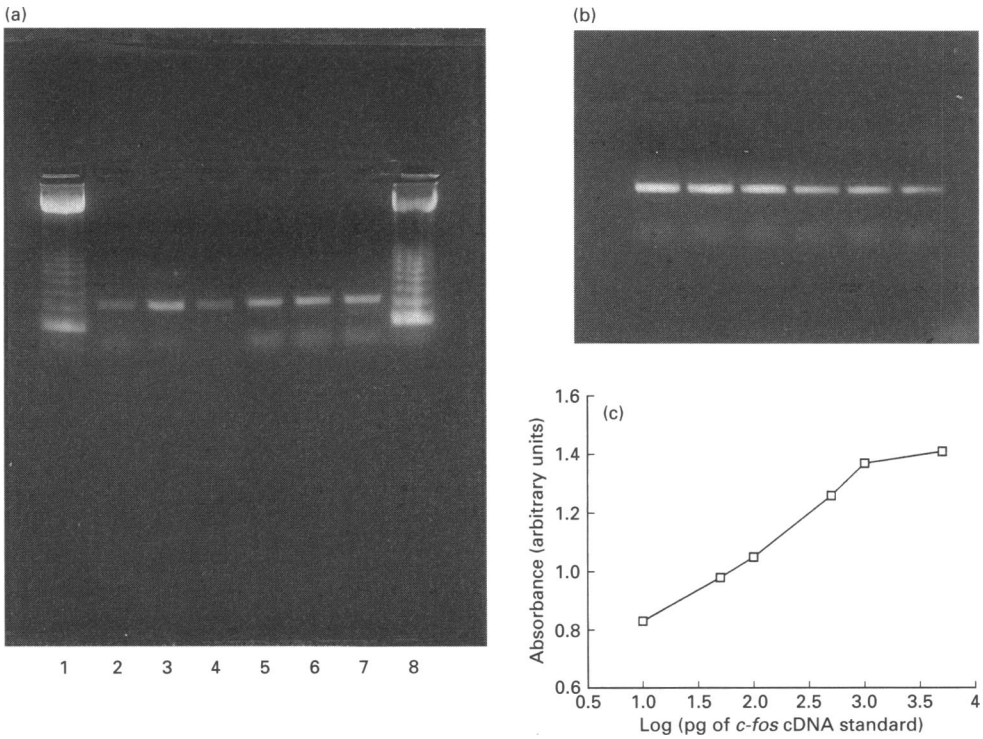

Figure 1 *c-fos* and *c-jun* PCR products and a PCR standard curve

(**a**) *c-fos* (left) and *c-jun* (right) PCR products. PCR products from experimental muscle samples were run on a 2% agarose gel stained with ethidium bromide. Discrete bands at 261 bp (*c-fos*; lanes 2, 3 and 4) and 267 bp (*c-jun*; lanes 5, 6 and 7) are visible. A 123 bp DNA ladder was run in lanes 1 and 8. (**b**) PCR products of *c-fos* standard cDNAs run on an ethidium bromide-stained agarose gel. (**c**) Plot of the absorbance of PCR product against the log of pg of *c-fos* cDNA standard.

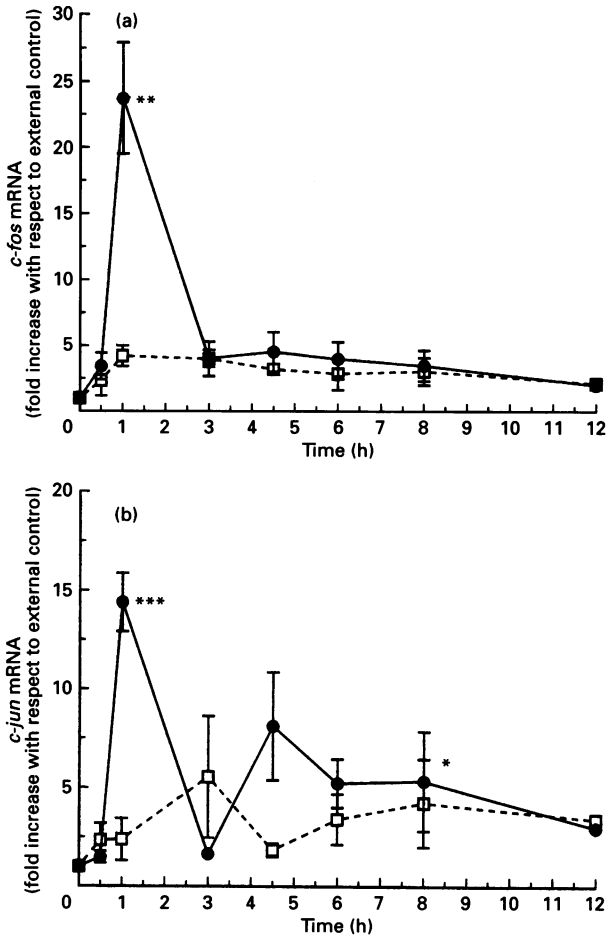

Figure 2 Time-course of *c-fos* (a) and *c-jun* (b) mRNA expression in the LD subjected to passive stretch

Data are presented for the experimental (●) and contralateral (□) muscles of three batches of animals. Means ± S.E.M. were calculated using Student's *t* test where *P* values refer to significant differences from sham-operated values: $^{*}P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.01$.

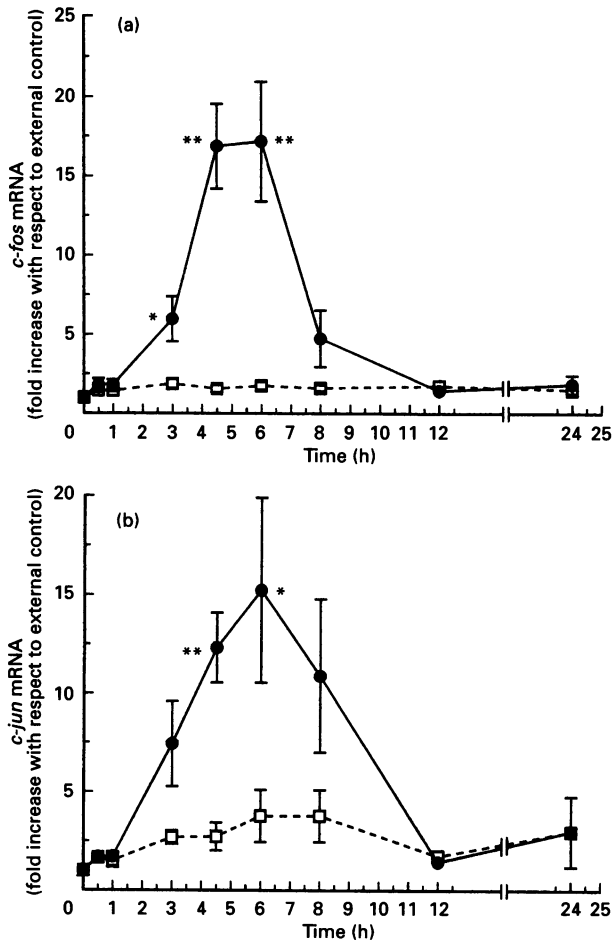

Figure 3 Time-course of *c-fos* (a) and *c-jun* (b) mRNA expression in the LD subjected to 10 Hz electrical stimulation

Data presented as in Figure 2.

approx. 4.5–6 h of initiating stimulation (Figures 3a and 3b). For both genes, the peaks were broader than those produced by stretch alone, although *c-jun* gave a broader message peak than *c-fos*. For both the stretch and electrical stimulation regimes, the magnitude of the fold increases for the two genes was similar and was of the order of 15- to 20-fold when compared with values for either external control or sham muscles. Values derived from the contralateral control muscles were only slightly elevated above external control values.

When stretch and electrical stimulation were combined, *c-fos* and *c-jun* mRNA were both expressed in a biphasic manner, with the peaks coinciding temporally with the peaks obtained for the individual regimes (Figures 4a and 4b). For *c-fos*, the secondary peak was smaller (around 7-fold that of the control) compared with the first peak (16-fold) at 1 h. The reverse was true, however, of the expression of *c-jun*. Here, the 4.5–6 h peak was around twice the magnitude of the earlier peak at 1 h. Once again, contralateral muscles gave values for *c-fos* and *c-jun* expression which were not statistically different from baseline values.

Competitive PCR, using specifically designed *c-fos* and *c-jun* competitors, was undertaken to obtain more accurate quantitative measurements on selected samples and to confirm the results given above (Table 1). One experimental sample (around peak expression) and one contralateral sample from each training regime was analysed for *c-fos* and *c-jun* mRNA. Results are again expressed as fold increases to enable direct comparisons with the external standard (semi-quantitative) method. Both PCR methods yielded fold increases which were very similar (see Table 1), thus validating our more extensive use of non-competitive RT-PCR using external standards.

Immunocytochemistry

The early, transient induction of *c-fos* and *c-jun* mRNA is accompanied by an accumulation of their respective protein products in the mechanically stimulated muscles. c-Fos and c-Jun immunoreactivity was barely detectable in sections of external control muscles (Figures 5a and 5b), even following prolonged incubation (up to 1 h) with the DAB substrate.

Sections from muscles which had undergone 10 Hz electrical stimulation for 6 h demonstrated c-Fos immunoreactivity almost exclusively in non-myofibre nuclei (Figure 5c). The distribution of c-Jun immunoreactivity in the same muscles largely corresponded to that seen with c-Fos, although a small proportion of myofibre nuclei stained positive for the former (Figure 5d). In contrast, sections from muscles which had been passively

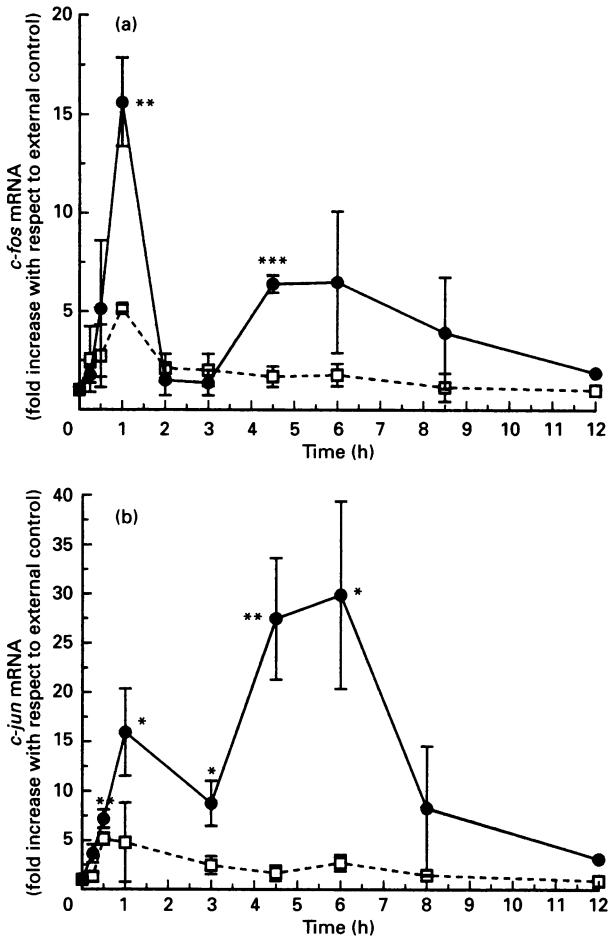

Figure 4 Time-course of *c-fos* (a) and *c-jun* (b) mRNA expression in the LD subjected to passive stretch plus 10 Hz electrical stimulation

Data presented as in Figure 2.

Table 1 Fold increases in *c-fos* or *c-jun* expression (compared with contralateral muscle): comparison of competitive and non-competitive PCR methods

For each regime, an experimental muscle sample and a sample from its contralateral muscle were analysed for *c-fos* and *c-jun* mRNA by competitive PCR (using a *c-fos* or *c-jun* mimic) and by non-competitive PCR (using external standards). Results are expressed as fold increases in the experimental samples compared with the contralaterals.

Regime	Samples [time (h)]	Fold increase: Competitive PCR	Fold increase: Non-competitive PCR
c-fos			
Stretch	1	6.3	5.5
Electrical stimulation	8	9.4	12.3
Combined	0.5	4.0	5.3
c-jun			
Stretch	1	2.2	2.4
Electrical stimulation	3	3.0	3.2
Combined	1	3.3	4.3

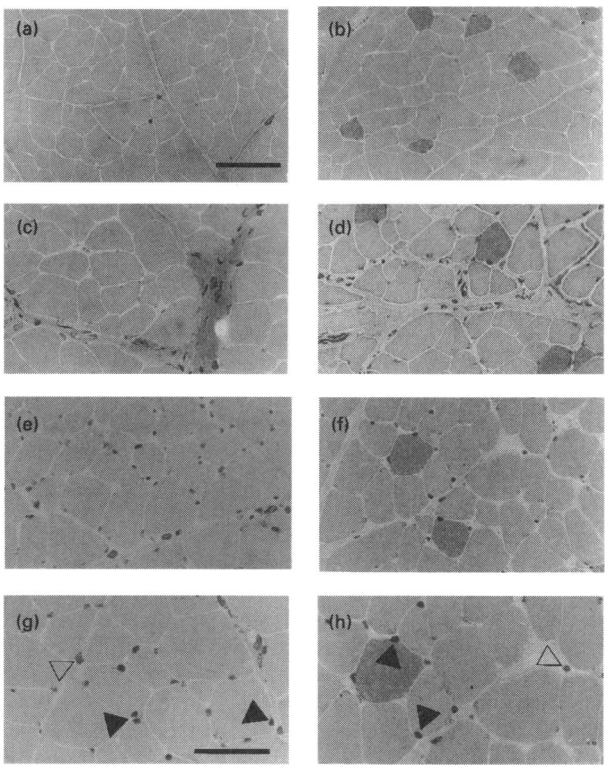

Figure 5 Cellular localization of Fos and Jun immunoreactivity in sections from control, electrically stimulated and stretched LD

All sections were cut to 7 μm and immunostained for either c-Fos (**a**, **c**, **e** and **g**) or c-Jun (**b**, **d**, **f** and **h**). These are representative sections of control (**a** and **b**), 4.5 h electrically stimulated (**c** and **d**) and 6 h stretched (**e** and **f**) muscles. Scale bar = 80 μm. Photographs (**g**) and (**h**) are higher magnifications of (**e**) and (**f**) respectively, allowing localization of immuno-positive nuclei relative to the plasma membrane of the fibres. Solid arrowheads show immunostaining within myofibres and open arrowheads show staining of interstitial cell nuclei. Scale bar = 60 μm.

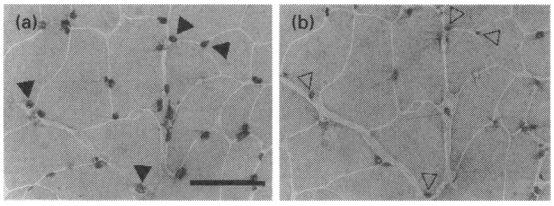

Figure 6 Co-localization of Fos and Jun immunoreactivity in serial sections from stretched LD

Serial sections were cut to 5 μm and alternately immunostained for either c-Fos (**a**) or c-Jun (**b**). Arrowheads show analogous, positively stained myonuclei in each section. Scale bar = 60 μm.

stretched for 4.5 h showed strong c-Fos and c-Jun immunoreactivity within nuclei of both myofibres and interstitial cells (Figures 5e and 5f). The localization of these cellular oncoproteins within the myofibres is more clearly seen at higher magnification (Figures 5g and 5h). Serial sections (4–5 μm) were also taken from stretched muscles to establish whether the c-Fos and c-Jun proteins could be co-localized within the same nuclei. Results shown in Figures 6a and 6b verify that Fos/Jun co-localization was detectable in a proportion of the myonuclei.

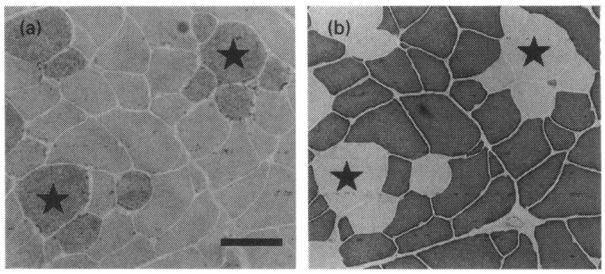

Figure 7 Staining of slow, oxidative fibres in control LD by the Jun polyclonal antibody

Serial sections were cut to 7 μm and alternately stained for either c-Jun (**a**) or myosin ATPase (**b**). Stars in (**a**) highlight fibres stained with the Jun antibody. These clearly correspond to the slow, oxidative fibres which are light-staining after a pre-incubation at pH 10.4 (**b**) (for methods see [19]). Scale bar = 50 μm.

Very similar patterns of c-Fos and c-Jun expression were seen in muscles which had been electrically stimulated for either shorter (i.e. 4.5 h) or longer (i.e. 8 h) periods than those in Figures 5c and 5d. Similar results to those shown in Figures 5(e) and 5(f) were obtained with muscles stretched for 3 or 6 h (results not shown).

Although we are unable to explain this phenomenon at present, the c-Jun antibody appeared to interact with a small proportion of the fibres in both control and experimental muscles (Figures 5b, 5d, 5f and 5h). Using a well established myosin ATPase histochemical technique (for methods see [19]) we have shown that the c-Jun polyclonal antibody also stained slow, oxidative fibres (Figures 7a and b). This staining, together with all the staining patterns for c-Fos and c-Jun in the experimental muscles, was abolished when the sections were incubated with non-immune sheep serum or with pre-absorbed primary antibodies (results not shown). The specificities of the primary antibodies were confirmed by detection of single bands on immunoblots of experimental LD muscle extracts (results not shown).

DISCUSSION

In this study we have demonstrated both the rapid induction of mRNA and nuclear accumulation of c-Fos and c-Jun gene products in skeletal muscle subjected to mechanical training *in vivo*, using either stretch alone, electrical stimulation at 10 Hz or a combination of both.

Although similarly co-expressed, the time-courses of the induction of *c-jun* mRNA were generally more complex than those of *c-fos*. Whereas *c-fos* was induced only transiently (see Figures 2a, 3a and 4a), *c-jun* mRNA was detected over longer time periods and apparently re-induced to provide a secondary peak in response to stretch (see Figures 2b, 3b and 4b). One possible explanation for these differences is that *c-jun* transcription is positively regulated by its own product [24], whereas c-Fos down-regulates its own expression [25]. This might also account for the increased magnitude of the second *c-jun* peak following stretch plus electrical stimulation (Figure 3c) and the decreased magnitude of the corresponding *c-fos* peak (Figure 2c). The picture is further complicated, however, by the fact that another member of the Jun family, Jun-B, negatively regulates *c-jun* expression [24] and other members of the Fos family undoubtedly have a role in regulating *c-fos* expression.

Electrical stimulation of the LD led to peak expression of *c-fos* and *c-jun* at around 4.5–6 h, i.e. at least 3 h after peak expression in response to the stretch stimulus. In a preliminary study, we have also shown that the patterns of expression reported here are similar to those found in a hind limb muscle, the extensor digitorum longus, following electrical stimulation or stretch by limb immobilization [26].

Upon combining stretch with electrical stimulation, biphasic expression of both *c-fos* and *c-jun* was induced; the timing of the two peaks coinciding with the responses to each individual stimulus and suggesting that stretch and electrical stimulation may trigger cellular oncogene expression via separate signalling pathways.

The use of immunocytochemistry enabled us to answer three important questions: (i) are the increases in *c-fos* and *c-jun* mRNA reflected in an accumulation of their respective protein products, (ii) in which cell type are the c-Fos and c-Jun proteins being expressed, and (iii) are these two proto-oncogenes co-expressed within the same nuclei. The results clearly show that following both electrical stimulation and stretch, the induction of *c-fos* and *c-jun* mRNA does give rise to increased levels of the corresponding proteins.

Although electrical stimulation of the LD resulted in prominent c-Fos and c-Jun immunoreactivity, the vast majority of these nuclei were associated with interstitial cells and not those of the myofibres. In keeping with observations *in vitro* [27], these changes in the interstitial cells are probably associated with cell proliferation. This would be consistent with the known rapid increase in capillary density following continuous electrical stimulation [13]. The activation of fibroblasts to divide and secrete more collagen may ultimately increase the passive resistance of these muscles, thereby affecting their mechanical performance. Another possibility involves the infiltration of macrophages, if the stimulation protocol causes any muscle damage or inflammation.

In contrast to these changes, passively stretching the LD muscle led to a rapid and transient peak expression of *c-fos* and *c-jun* message after 1 h (Figure 2). Fos and Jun proteins were clearly localized within nuclei of the myofibres, as well as interstitial cells (Figure 5). Hence, stretch activates the myonuclei and/or the satellite cells and induces muscle fibre hypertrophy [11,28].

A few studies have attempted to investigate the possible role of proto-oncogenes in the induction of cardiac hypertrophy. For example, Schunkert et al. [29] reported similar temporal changes in cardiac muscle *c-fos* and *c-jun* message levels to those reported here when the wall stress of the heart was increased during systole. However, in pressure-overloaded hearts, *c-fos* expression was found to be maximal somewhat later after 3–8 h [4,30]. An accumulation of Fos protein has also been demonstrated in the myocyte nuclei, with minimal staining of fibroblasts and vascular smooth muscle, of isolated rat hearts subjected to acute pressure overload [29]. c-Myc has also been localized in cardiomyocytes following haemodynamic overload *in vivo* [31]. However, the acute pressure changes imposed on the ventricles in such studies could conceivably cause myocyte damage. If so, this could complicate the interpretation of these findings as *c-myc* mRNA levels are known to rise transiently in association with the damage inflicted on the fibres of the anterior LD muscle of the chicken [32].

Two important findings of this study were the co-expression of *c-fos* and *c-jun* mRNA (Figure 2) and the co-localization of Fos and Jun proteins within the myonuclei (Figure 6) of the stretched LD. Even larger numbers of Fos/Jun positive nuclei might have been detected had it been possible to cut even thinner serial sections (< 3 μm). Although still speculative at this stage, our hypothesis is that these two proto-oncogenes, probably via the AP-1 transcription factor, activate other growth-related genes,

which in turn initiate muscle fibre hypertrophy through an autocrine/paracrine action. We know, for example, that insulin-like growth factor-1 message levels are markedly elevated in association with the rapid hypertrophic response to stretch [18,28]. When examined histologically, the same stretched muscles showed a small increase in the connective tissue content around some muscle fibres, but the fibres themselves were undamaged. Therefore, the localization of Fos and Jun proteins within the nuclei of these stretched post-mitotic fibres suggests that these oncoproteins may play an important role in the early induction of muscle hypertrophy. This contrasts with the almost total absence of nuclear staining for these oncoproteins in the electrically stimulated muscles (Figures 5c and 5d), whose fibres undergo dramatic atrophy and not hypertrophy [16]. Also, in sharp contrast to electrical stimulation, passive stretch does not change the metabolic and contractile properties of the muscle fibres (R. James, J. D. Altringham, I. S. Young, V. M. Cox and D. F. Goldspink unpublished work). Taken together, this suggests that while these genes may be important in inducing fibre hypertrophy, they are unlikely to be directly involved in initiating the well-established changes in the phenotypic properties of muscles when subjected to chronic electrical stimulation [13].

The present results indicate that under conditions known to induce muscle hypertrophy *in vivo* (i.e. stretch), *c-fos* and *c-jun* are activated in the nuclei of post-mitotic fibres. Although the same genes are activated by electrical stimulation, this mainly occurs in cells of the interstitial tissue and the fibres themselves undergo atrophy [16]. Thus, the expression of these two proto-oncogenes differ in both their timing and cellular localization following two different types of mechanical stimulation.

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