

# Stress-dependent and -independent expression of the myogenic regulatory factors and the *MARP* genes after eccentric contractions in rats

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The relationship between muscle mechanical conditions and gene expression was investigated by varying both stress and contraction mode imposed upon rat dorsiflexors ( $n = 25$ ), activating them at high or low frequencies (150 Hz or 40 Hz) either eccentrically or isometrically. Muscle physiological, immunohistochemical and gene expression changes were then measured 24 h after the exercise bout. Peak stress was the best predictor of muscle injury, independent of contraction mode (i.e. eccentric or isometric). When peak stresses were matched, no physiological or immunohistochemical differences were detected between isometric and eccentric contractions. The expression of certain myogenic regulatory and muscle ankyrin repeat protein (*MARP*) genes (*myoD*, *myogenin*, *MLP* and *CARP*) depended both on peak muscle stress achieved during contraction and contraction mode. In contrast, *Arpp/Ankrd2* was dramatically upregulated only by eccentric contractions, but not by isometric contractions, even though the stress level of the eccentric contractions varied over a three-fold range and overlapped with that of the isometric group. The role that *Arpp/Ankrd2* upregulation plays in the biological response to eccentric contraction remains to be determined, as does the control mechanism whereby the expression of certain genes (such as *myoD*, *myogenin*, *MLP* and *CARP*) is sensitive to muscle stress while another (*Arpp/Ankrd2*) is sensitive only to contraction mode.

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Skeletal muscle tissue is highly plastic (Pette, 1990). A plethora of reports describe consistent and systematic changes in muscles subjected to altered use patterns such as immobilization (Booth, 1982), endurance exercise (Saltin & Gollnick, 1983), eccentric exercise (Morgan & Allen, 1999), weightlessness (Edgerton & Roy, 2000), tenotomy (Jamali *et al.* 2000) and chronic electrical stimulation (Salmons & Henriksson, 1981). Muscles may change their fibre size, fibre type, microcirculation, calcium regulation, mitochondrial density and calcium transport, in order to adapt to their new functional requirements. Such alterations result from orchestrated gene expression patterns both within and around the muscle cell. While numerous descriptive studies describe genes that are sensitive to muscle's general mechanical environment, there are almost no data available that attempt to define the fundamental mechanical variables (i.e. stress, strain, energy absorbed) transduced by skeletal muscles. Strain-based mechanisms might implicate such cellular components as strain-activated ion channels (Guharay

& Sachs, 1984; Komuro *et al.* 1991), while stress-based mechanisms may implicate the involvement of cytoskeletal elements, membrane-associated G-proteins or integrins (Frangos *et al.* 1985; Ingber, 1990; Banes, 1993; Petrof *et al.* 1993). In any case, defining both the mechanism of tissue mechanical transduction and the genes affected by specific mechanical factors is of tremendous importance.

Certain genes and gene families have been demonstrated as being highly mechanically sensitive in striated muscle and are excellent candidates for involvement in the adaptive response to mechanical perturbation. The recently discovered *MARP* family, or muscle ankyrin repeat proteins, (cardiac ankyrin repeat protein (*CARP*), *Arpp/Ankrd2*, and diabetes-associated ankyrin repeat protein (*DARP*), respectively), have been shown *in vivo* and *in vitro* to be highly responsive to muscle mechanical status (Kemp *et al.* 2000; Miller *et al.* 2003). In an *in vivo* model, both *CARP* and *Arpp/Ankrd2* rapidly reached peak expression levels of ~10 times contralateral values 12–24 h after the eccentric contraction (EC) bout

and returned to baseline levels within 72 h (Barash *et al.* 2004). A second protein, muscle LIM protein (MLP), is a Z-disk protein that was recently identified as also being dramatically upregulated in response to EC-induced muscle injury (Chen *et al.* 2002; Barash *et al.* 2004). MLP has been found in the nucleus during early development (Arber *et al.* 1994), where it is a potent activator of the myogenic regulatory factor myoD (Kong *et al.* 1997). This nuclear activity suggests that MLP may have transcriptional activity in addition to its structural role, leading to the intriguing possibility that MLP is a striated muscle stress sensor (Knöll *et al.* 2002). Unfortunately, there have been no studies in which expression of these novel gene products have been measured under controlled mechanical conditions.

Skeletal muscle tissue, being contractile in nature, affords the unique opportunity to vary tissue stress systematically, simply by altering activation frequency and exploiting the well-defined relationship between force and frequency (Close, 1972). Further, because the force

response of skeletal muscle is highly asymmetrical when comparing active shortening to active lengthening (Katz, 1939), muscle contraction mode (i.e. active lengthening, active shortening or isometric contraction) can also be exploited to alter tissue stress experimentally. With the development of minimally invasive animal exercise models (McCully & Faulkner, 1985; Ashton-Miller *et al.* 1992; Caiozzo *et al.* 1992; Lieber *et al.* 1994; Peters *et al.* 2003; Barash *et al.* 2004), it is now possible to modulate muscle stress and activation mode precisely in an *in vivo* model. Thus, the purpose of this study was to impose controlled stresses and contraction modes upon muscles *in vivo*, in order to understand the relationship between mechanical factors, the resulting injury and the subsequent gene expression pattern. A brief version of this study has appeared in abstract form (Hentzen *et al.* 2004).

## Methods

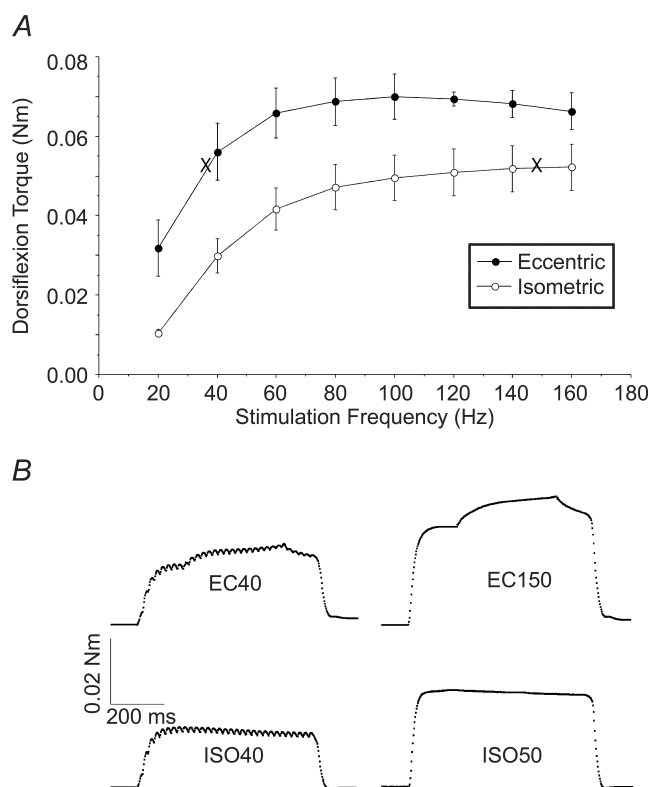
### Animal care

Laboratory animals used in this study were untrained, adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA), with an average size of  $443 \pm 24$  g (mean  $\pm$  s.d.,  $n = 29$ ). Rats were housed two per cage at 20–23°C with a 12 h dark–light cycle. All procedures were approved by the University of California and VA Medical Center Committees on the Use of Animal Subjects in Research. After terminal experiments, animals were killed with an intracardiac injection of pentobarbitol sodium (0.5 ml of 390 mg ml<sup>-1</sup> solution).

### Experimental protocol

Animals were anaesthetized using 0.3 ml subcutaneous injection of a ketamine–rompum–acepromazine mixture (25, 2.5, and 0.5 mg ml<sup>-1</sup>, respectively), and maintained on isoflurane anaesthesia (2%, 1.5 l min<sup>-1</sup>) throughout the experiment. Respiratory rate was monitored visually, and anaesthetic concentration was adjusted as needed. Ankle isometric dorsiflexion torque was measured prior to eccentric activation or isometric activation as well as 24 h later. The exercise bouts consisted of 30 contractions (either eccentric or isometric), with 2 min between each contraction to eliminate the effects of fatigue, as previously described in detail (Peters *et al.* 2003).

A small lateral incision was made near the fibular head, and the peroneal nerve was isolated as previously described (Peters *et al.* 2003). This method permitted measurement of isometric dorsiflexion torque with a coefficient of variation of repeated measures between two different measuring days of  $7.3\% \pm 3.5\%$  (pilot study of eight animals). Rats ( $n = 4$ ) were stimulated at frequencies ranging from 5 to 150 Hz to determine the torque–frequency relationship either isometrically (○, Fig. 1A) or eccentrically (●, Fig. 1A). This was performed



**Figure 1. Determination of experimental parameters matching torque across contraction modes**

A, torque–frequency relationship for muscles stimulated either isometrically (○) or eccentrically (●) across a range of frequencies. Data represent the average  $\pm$  s.e.m. of  $n = 4$  animal subjects. The crosses illustrate the points chosen to match stress between isometric and eccentric contractions (EC40 and ISO150). B, sample contractile records of muscles stimulated under the four different conditions described in Methods. Note that nearly identical peak torque is obtained when stimulating eccentrically at 40 Hz (EC40) and isometrically at 150 Hz (ISO150).

to determine stimulation frequencies and modes that result in identical stimulation stresses (see sample records in Fig. 1B). (The small decrease in peak torque for the eccentric contractions in Fig. 1A at high frequency probably represents a slight degree of injury that occurs with repeat torque–frequency testing in the eccentric mode).

After treatment, animals recovered for 24 h, at which time isometric dorsiflexion torque measurement was repeated. Animals were sacrificed and the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were excised, immediately frozen in liquid nitrogen-cooled isopentane ( $-159^{\circ}\text{C}$ ) and stored at  $-80^{\circ}\text{C}$  for subsequent analysis. This time period was chosen based on previous studies that documented near-peak expression of both the myogenic regulatory factors (Peters *et al.* 2003) and MARP proteins (Barash *et al.* 2004) at this time point. While other gene expression profiles were increased 2–5 days after the stimulation bout (Peters *et al.* 2003), we were interested in the early events that presumably would be most closely related to the cellular transduction of the mechanical events.

### Experimental design

To determine whether muscle stress acts as an independent variable or whether treatment mode (eccentric *versus* isometric) was a significant factor in the muscle's response, an experimental design was created that varied both the treatment mode and muscle stress. A high-stress, eccentric mode was created by treating animal subjects with eccentric contractions while stimulating the peroneal nerve at 150 Hz (EC150,  $n = 5$ ). Two groups, matched for peak stress (crosses on lines in Fig. 1A) but 'exercised' in different modes were created by treating with either eccentric contractions at 40 Hz (EC40,  $n = 7$ ) or isometric contractions at 150 Hz (ISO150,  $n = 6$ ). As a control for stimulation frequency, and to balance the experimental design, a group of subjects treated isometrically at 40 Hz was also generated (ISO40,  $n = 7$ ). Ideally, the experimental design would have also included a group subjected to shortening contractions at the same stresses. However, based on the dramatic muscle force drop that occurs during shortening, this experimental group was impossible to create at any stimulation frequency or shortening velocity. Thus, in this report, 'contraction mode' refers to either eccentric or isometric contraction. For immunohistochemistry and transcript assays, control TA muscles were obtained from rats that had not undergone either eccentric or isometric treatment protocols.

Contractile properties were measured as previously described (Peters *et al.* 2003). Briefly, isometric dorsiflexion torque was measured by activating the peroneal nerve maximally using a 15 V stimulus, with a train duration of 650 ms. Muscles were stimulated

at 2 min intervals at frequencies of 5, 15, 25, 40, and 100 Hz, to determine the torque–frequency relationship. Maximum isometric torque was defined as dorsiflexion torque at 100 Hz stimulation frequency, which was always the greatest value achieved. Eccentric contractions were induced by imposing a 38 deg ankle plantarflexion over a 400 ms duration during maximal dorsiflexor activation. All activations were repeated at 2 min intervals for 60 min, yielding 30 contractions in the prescribed mode and at the prescribed frequency.

### Immunohistochemistry

Muscle cross-sections (10  $\mu\text{m}$  thick) were taken from the midbelly of the TA only, since the EDL did not previously show any abnormalities (Peters *et al.* 2003). A portion of the muscle cross-section, free from sectioning artifacts, excessive connective tissue or large blood vessels, was labelled with monoclonal antibodies to desmin (DER11, 1:1000, Novocastra Laboratories, Newcastle, UK), fibronectin (EDA domain, 1:50, Sigma), vimentin (VIM-V9, 1:5000, Novocastra), embryonic myosin heavy chain (clone 1.652, 1:10, courtesy of Helen Blau, Stanford University), or laminin (clone II (2E8), 1:1000, Gibco, Carlsbad, CA, USA) using an indirect immunoperoxidase technique (Vectastain Elite ABC Kit, Vector laboratories, Burlingame, CA, USA). Muscle sections were treated with bovine serum albumin (Sigma, St Louis, MO, USA) as a blocking agent, and incubated with the primary antibodies overnight. The secondary antibody was biotinylated, rat-adsorbed, horse antimouse (1:20,000, Vector), and labelling was visualized using diaminobenzidine (Sigma).

Cross-sections were viewed at 25 $\times$  magnification, to enable viewing the entire muscle cross-section, and affected fibres were counted directly by an observer blinded to tissue experimental group. Each affected fibre of every section was counted to quantify immunohistochemical appearance, and the criteria for 'abnormal' fibres were exactly as previously described (Peters *et al.* 2003) and illustrated in Fig. 1 of that reference.

### Skeletal muscle transcript levels

Total RNA was isolated from frozen muscle tissues using TRIZOL extraction (Gibco BRL, Grand Island, NY, USA). RNA concentration was measured by absorption at 260 nm, and separation of RNA from contaminant genomic DNA and protein was checked based on a 260 nm/280 nm absorption ratio of  $\sim 2.0$ . One microgram of RNA per reaction was reverse transcribed (Superscript, Invitrogen, Carlsbad, CA, USA) into cDNA using Oligo dT as the primer and the manufacturer's protocol, and thus all transcript levels are expressed per microgram of extracted RNA. Primers were designed to amplify *myostatin*, *MyoD*, *myogenin*, *MLP* (also referred to as *CRP3*), *CARP* and *Arpp/Ankrd2* in the translated region, while for *embryonic*

**Table 1. Primers used for quantitative real-time PCR\***

Transcript	Sense primer	Antisense primer
<i>MLP</i> (X81193, 213 bp)	5'-ATCAGAGAAGTGCCACGATG-3'	5'-GTAAGCCCTCCAAACCAAT-3'
<i>CARP</i> (L81174, 270 bp)	5'-AGGACCGGTCACTACGAGTGT-3'	5'-AGCTATGCGCGAGTTTTTGT-3'
<i>Arpp/Ankrd2</i> (XM_219881, 226 bp)	5'-CGGGATCCAGAACCTCATAGA-3'	5'-CAGTGCTGTCCGACGGAAGCTC-3'

\*GenBank accession number and PCR product length given in parentheses after each transcript name.

*MHC* the sense primer was designed in the translated region and the antisense primer in the 3'-untranslated region, where the sequence is more specific for this *MHC* isoform (Periasamy *et al.* 1985; Weiss *et al.* 1999). Primers for *myostatin*, *MyoD*, *myogenin* and *embryonic MHC* were as previously described (Peters *et al.* 2003) while primers, accession numbers and expected product lengths for *MLP*, *CARP* and *Arpp/Ankrd2* are provided in Table 1.

Quantification of transcript levels was performed using the Cepheid SmartCycler (Sunnyvale, CA) on cDNA, as previously described in detail for *myostatin*, *MyoD*, *myogenin* and *embryonic MHC* (Peters *et al.* 2003). For *MLP*, *CARP* and *Arpp/Ankrd2*, 1  $\mu$ l of 1:10 diluted cDNA was analysed using platinum taq DNA polymerase (Invitrogen) and SYBR Green dye. All samples were analysed in triplicate along with the gel-purified PCR product standard. The PCR reaction vessel (25  $\mu$ l) contained: 2.5  $\mu$ l 10 $\times$  PCR buffer (1 $\times$ ), 2 mM MgCl<sub>2</sub>, 0.2  $\mu$ M sense and antisense primers, 0.2 mM dNTP, 0.2 $\times$  SYBR Green and 1 U platinum taq polymerase. Two-step amplification was performed as follows: An initial hold at 95°C for 3 min (*myogenin*) or 5 min (for *MLP*, *CARP*, *Arpp/Ankrd2*, *myostatin*, *MyoD* and *embryonic MHC*) was followed by 40 cycles of denaturing at 95°C for 15 s, and annealing/extension at 68°C for 30 s (*CARP*), 66°C for 30 s (*Arpp/Ankrd2*), 62°C for 40 s (*myostatin*), 67°C for 40 s (*MyoD*), 68°C for 30 s (*myogenin*) or 69°C for 40 s (*MLP* and *embryonic MHC*). Success of each reaction was deduced based on observing a single reaction product on an agarose gel and a single peak on the DNA melting temperature curve determined at the end of the reaction.

### Analysis of experimental data

Mean values among treatment groups were compared by one-way analysis of variance (ANOVA). *Post hoc* multiple comparisons between each time period and, in some cases, control groups were made using Fischer's least squared differences (LSD) test. Correlations between and among variables were calculated using simple linear regression and multiple linear regression. Stepwise regression and factor analysis were the multivariate methods used to understand independent relationships among the variables measured.

Two separate indicators of tissue stress were obtained from the real-time torque measurements: torque-time integral and peak torque. The total torque-time integral for each subject was calculated according to the formula:

$$\text{Torque - time integral} = \sum_{i=1}^{30} \int_0^{650} \tau_i(t)$$

where  $\tau_i(t)$  represents dorsiflexion torque at time  $t$  (in ms) during a contraction, the subscript  $i$  is contraction number (from 1 to 30), the total stimulation time per contraction was 650 ms, and the time interval between data points was 0.25 ms (i.e. data acquisition rate of 4000 Hz). (Torque-time integral was used for this study instead of the traditional value of energy absorbed (Fung, 1981; Sam *et al.* 2000) since, for the isometric contractions, no external work was performed and thus energy absorbed for isometric contraction would be zero.) Peak torque during treatment was defined as the peak torque measured for any of the 30 contractions, regardless of the contraction number. In practice, peak torque was observed within the first five contractions for the eccentric bouts, but ranged from the first to last contraction for either isometric treatment.

Statistical significance level ( $\alpha$ ) was set to 0.05 and statistical power ( $1 - \beta$ ) exceeded 80% in all cases where results were not significantly different, demonstrating an adequate sample size. All analyses were performed with the StatView program (v.5.1, Abacus Concepts, Berkeley, CA, USA). Data are presented in the text as mean  $\pm$  s.e.m. unless otherwise stated.

## Results

### Mechanics of treatment

Dorsiflexion torque was recorded in real-time for each contraction of each bout as described in Methods. The torque-time integral varied significantly among groups ( $P < 0.0001$ ) with the highest value observed for the EC150 group ( $1.32 \pm 0.04$  N m s) and the lowest for the ISO40 group ( $0.67 \pm 0.07$  N m s). While the goal of the experimental design was to match peak torque of the EC40 and ISO150 groups, the ISO150 group ( $1.17 \pm 0.07$  N m s) had a significantly greater torque-time integral compared to the EC40 group ( $0.99 \pm 0.04$  N m s;  $P < 0.05$ ). As expected, dorsiflexion torque was initially highest for

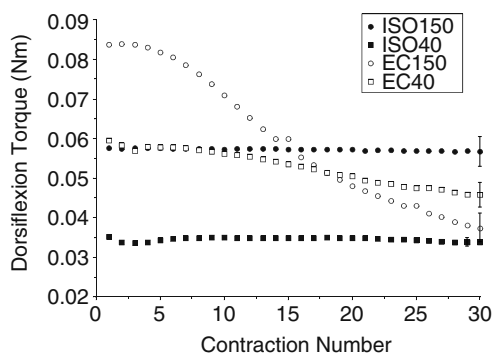
the EC150 group, lowest for the ISO40 group and intermediate for the EC40 and ISO150 groups (Fig. 2). Interestingly, while torque was nearly identical for the first ~10 contractions of the EC40 and ISO150 groups, it then dropped significantly for the EC40 group. This formed the basis for the significant difference in torque–time integrals between the EC40 and ISO150 groups.

Peak torque during treatment varied significantly among the four experimental groups (Fig. 3,  $P < 0.0001$ ). Not surprisingly, the EC150 group experienced the highest peak torque ( $0.086 \pm 0.002$  N m) and the ISO40 group the lowest ( $0.036 \pm 0.001$  N m), while no significant difference was observed between the EC40 ( $0.061 \pm 0.002$  N m) and ISO150 groups ( $0.056 \pm 0.003$  N m,  $P > 0.3$ ). Thus, in spite of differences in torque–time integrals, our effort to match peak stress between the EC40 and ISO150 groups was successful.

### Torque decline after treatment

Two-way ANOVA revealed a significant torque decrease after treatment ( $P < 0.01$ ), and a significant time–treatment interaction ( $P < 0.01$ ). When expressed as a percentage change relative to initial torque level, the largest torque change was observed for the EC150 group ( $-32 \pm 2\%$ ), while the ISO40 demonstrated no change ( $+3.4 \pm 4.4\%$ ). Importantly, no significant difference was observed between the ISO150 ( $-9.7 \pm 6.1\%$ ) and EC40 ( $-14.4 \pm 5.8\%$ ) groups, suggesting similar degrees of physiological injury in response to these treatments ( $P > 0.4$ ), even though the EC40 group demonstrated a torque drop during the EC bout while the ISO150 group did not (Fig. 2).

When analysing correlations among physiological variables, the strongest predictor of the percentage change in torque 24 h after treatment was peak torque



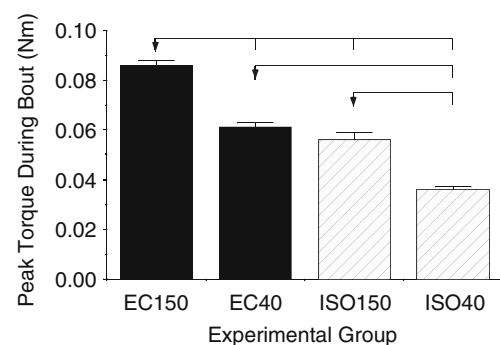
**Figure 2. Torque production records for the four treatment groups**

Each point represents the mean value for the group. s.e.m. bars are shown only for the final contraction for all groups for clarity. No change in data variability were seen as a function of contraction number. (○) EC150 (□) EC40 (●) ISO150 (■) ISO40.

achieved during treatment ( $r^2 = 0.58$ ,  $P < 0.0001$ ), while no significant correlation was observed between the torque–time integral and percentage change in torque 24 h after treatment ( $r^2 = 0.04$ ,  $P > 0.6$ ). These data suggest that the peak torque imposed upon the muscle during treatment, regardless of mode, was the best predictor of the magnitude of physiological ‘injury’ observed. In addition, the torque–time integral is not an indicator of the magnitude of injury experienced by the muscle. Factor analysis performed on all physiological variables (peak torque during treatment, torque before treatment, torque immediately after treatment (absolute and percentage), torque 24 h after treatment (absolute and percentage), and torque–time integral) supported this concept because only three factors emerged ( $P < 0.0001$ ,  $\chi^2 = 570$ , d.f. = 90). In this ‘physiological’ analysis, the EC40 and ISO150 groups were indistinguishable based on any mechanical variable measured.

### Immunohistochemical changes after treatment

Based on the physiological changes observed above, the results of the immunohistochemical analysis were not surprising (Table 2). Several proteins that are commonly used as markers of muscle injury were probed. The most severe indications of injury were observed for the EC150 group, and least severe indications were observed for the ISO40 group. No significant difference was observed between the EC40 and ISO150 group for any immunostaining pattern quantified (Table 2). A high correlation between peak torque during treatment and all immunohistochemical indicators of ‘injury’ was observed ( $r^2$  values ranging from 0.61 to 0.84). Again, factor analysis



**Figure 3. Peak torque measured during treatment in each of the various modes**

The largest torque change 24 h after eccentric contraction was observed for the EC150 group ( $-32 \pm 2\%$ ) while the ISO40 demonstrated almost no change ( $+3.4 \pm 4.4\%$ ). Importantly, no significant difference was observed between the ISO150 ( $9.7 \pm 6.1\%$ ) and EC40 ( $-14.4 \pm 5.8\%$ ). Symbols at the top of the figure indicate significant differences between leftmost group (with arrowhead) and another group when connected with a vertical bar ( $P < 0.05$ ). Error bars are s.e.m.

**Table 2. Number of cells per section with immunohistochemical abnormalities\***

Experimental group	Desmin negative	Vimentin positive	Fibronectin positive	Embryonic MHC positive
EC40	63.5 ± 23.0	26.2 ± 8.3	33.0 ± 14.4	51.8 ± 11.1
EC100†	219 ± 33	153 ± 11	477 ± 74	40.8 ± 18.1
EC150	629 ± 102	409 ± 55	518 ± 94	90.0 ± 31.0
ISO40	0.6 ± 0.42	2.8 ± 1.4	1.0 ± 0.61	28.5 ± 7.4
ISO150	50.2 ± 27.3	18.2 ± 5.0	31.8 ± 15.6	34.0 ± 12.0
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.0 ± 5.1

\*Data presented are mean ± s.e.m. from each experimental group ( $n = 4-6$  per group) measured 24 h after the bout. For all immunostains except embryonic MHC, multiple  $t$  tests revealed EC150 > EC40, ISO150 and ISO40; EC40 = ISO150, and ISO40 < ISO150, EC40 and EC150 ( $P < 0.01$ ). No significant differences were observed between groups for embryonic MHC, since expression of this protein does not reach its peak level of 600–700 fibres until 5–7 days after an EC bout (Peters *et al.* 2003). Control muscles were not included in the statistical analysis due to zero variability for three of the four stains. †Values from Peters *et al.* (2003) are provided for comparison, which also includes a complete description of control values for these immunostains. Average number of muscle fibres in these sections is ~15 000 (Peters *et al.* 2003).

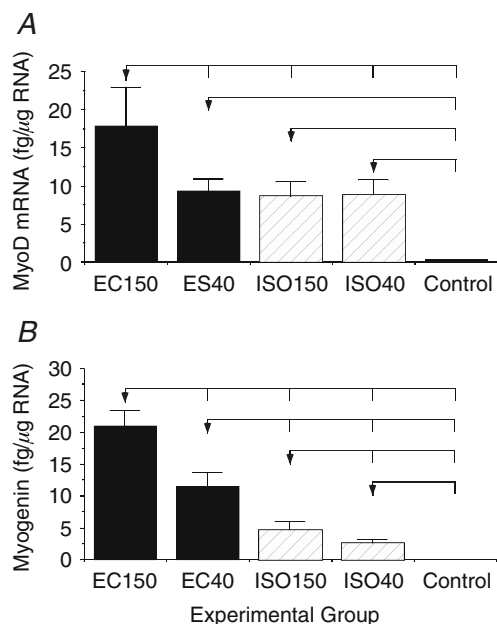
performed on all immunohistochemical indicators of injury revealed only three distinct factors ( $P < 0.0001$ ,  $\chi^2 = 622$ , d.f. = 90) indicating, as above, that the EC40 and ISO150 groups were indistinguishable based on any immunohistochemical assay used.

### Gene expression after treatment

The current experimental design was intended to investigate the early changes after treatment, especially

with regard to gene expression. The earliest changes observed previously were those associated with the myogenic regulatory factors (Peters *et al.* 2003) and, in an analogous mouse model, the *MARP* and *MLP* genes (Barash *et al.* 2004). To determine the similarity between the models, the time-course of *MARP* and *MLP* mRNA expression was measured on the muscles previously injured by a single eccentric bout at 100 Hz (Peters *et al.* 2003) with similar results to those previously reported for the mouse. Thus the two models are physiologically and biologically comparable (See Fig. 2A–D of Barash *et al.* 2004).

Gene expression patterns, unexpectedly, revealed sensitivity to both treatment mode and treatment stress, in spite of the fact that physiological and immunohistochemical responses were consistent across treatment groups and uniformly showed equivalent effects of EC40 and ISO150 treatments (the two groups treated at similar stresses). For example, *myoD* expression was significantly elevated in all treatment groups relative to controls (Fig. 4A) but expression in the EC150 group was significantly elevated above all other groups ( $P < 0.001$ ), none of which were significantly different from one another ( $P > 0.6$ ). In fact, the *myoD* expression level measured in the EC150 group was the highest level ( $17.9 \pm 5.0$  pg ( $\mu\text{g RNA}$ )<sup>-1</sup>) that we have measured in any experiment of this type (Peters *et al.* 2003). The other myogenic regulatory factor, *myogenin*, showed a graded response whereby eccentric groups were significantly different from isometric groups ( $P < 0.005$ ), and the EC150 and EC40 groups were significantly different from one another ( $P < 0.01$ , Fig. 4B). The most interesting comparison was between the EC40 and ISO150 groups, which, in spite of being subjected to similar peak stresses and demonstrating similar physiological and immunohistochemical responses (see above) yielded significantly different levels of the *myogenin* transcript ( $P < 0.05$ ; Fig. 4B).



**Figure 4. Expression of the myogenic regulatory factors 24 h after treatment**

A, *MyoD* gene expression levels. B, *myogenin* gene expression levels. Control *myogenin* levels were finite and easily measurable ( $0.057 \pm 0.004$  fg. ( $\mu\text{g RNA}$ )<sup>-1</sup>) but not visible on this graph's scale. Symbols at the top of the figure indicate significant differences between leftmost group (with arrowhead) and another group when connected with a vertical bar ( $P < 0.05$ ). Error bars are s.e.m.

Two of the members of the MARP family (Miller *et al.* 2003) showed distinct gene expression patterns in response to the various treatments. CARP was significantly elevated in all eccentric groups (Fig. 5A) compared to isometric groups with the EC40 group having a significantly lower expression level compared to EC150 group (Fig. 5A,  $P < 0.05$ ). CARP levels in both the ISO150 and ISO40 groups were slightly above levels measured in contralateral control or true experimental control muscles (Fig. 5A). Surprisingly, Arpp/Ankrd2 expression showed a distinct and repeatable association only with the mode of treatment, and no apparent dependence on tissue stress (Fig. 5B). Eccentric groups expressed Arpp/Ankrd2 at twice the level of both isometric groups, with no significant difference observed within either eccentric or isometric groups ( $P > 0.8$ ).

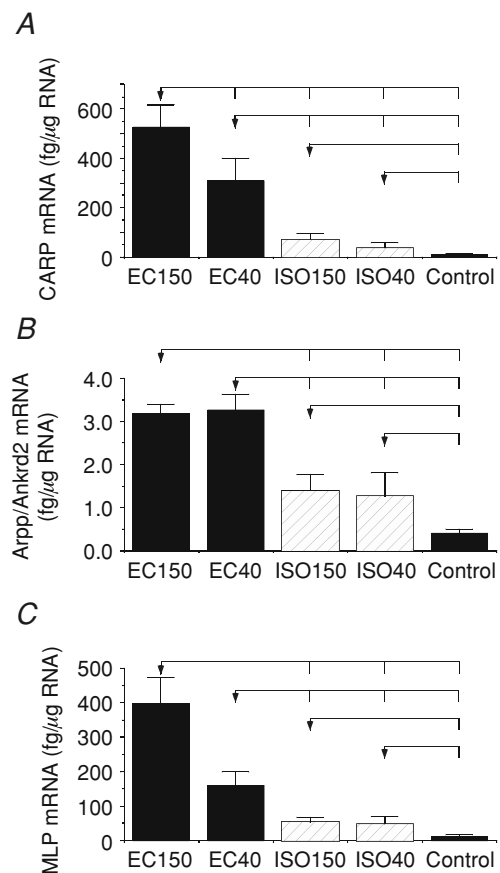
The MLP expression pattern roughly paralleled the CARP expression pattern (Fig. 5C), with the EC150 group expressing MLP at a significantly higher level compared to the EC40 group ( $P < 0.001$ ) which expressed MLP at significantly higher levels than either the ISO150 or ISO40 groups ( $P < 0.001$ ). No significant difference among experimental groups was observed for myostatin ( $P > 0.7$ ) or embryonic MHC ( $P > 0.2$ ) expression. This was probably based on the fact that the 24 h time period was too late to detect myostatin expression changes, and too early to detect embryonic MHC expression (Peters *et al.* 2003).

### Multivariate analysis

In an attempt to determine unique relationships among variables investigated in this study, stepwise regression was applied to the data set. Stepwise regression was used to predict the percentage torque drop 24 h after treatment. Surprisingly, of the 13 independent variables that *could* enter the regression equation, three physiological parameters, four immunohistochemical variables, and six gene expression levels), only one variable did – peak torque during the exercise bout ( $F$ -to-enter = 36.6  $F$ -to-enter represents the discriminating ability of a variable with higher  $F$ -values representing greater discriminating ability). While 11 of the independent variables initially had an  $F$ -to-enter that exceeded the threshold of 4.000 (all but *myoD* and *embryonic MHC* mRNA levels), these values all dropped to less than 3.000 after entry of the peak torque variable into the model. Such a uniform drop in  $F$ -to-enter indicates that percentage torque drop 24 h after treatment was highly correlated with all other variables measured but most highly correlated with peak torque.

Of the various transcript levels measured, myogenin expression varied most significantly among groups (Fig. 4B), and was thus another interesting candidate for stepwise regression analysis. In this case, several

variables entered the model and it is instructive to observe the process by which model entry occurred. In the first step of this analysis (Table 3), 9 of the 13 variables exceeded the  $F$ -to-enter, but the highest value was observed for the number of desmin-negative fibres ( $F$ -to-enter = 46.7) following closely by the number of cells that included fibronectin ( $F$ -to-enter = 44.2). The ‘desmin-negative’ variable alone accounted for 73% of the experimental variability (i.e.  $r^2 = 0.86^2$ ). In the second step, after accounting for covariation with the number of desmin-negative fibres (Table 4), *Arpp/Ankrd2* transcript level was one of only two variables of the remaining 12 with an  $F$ -value that would permit it to enter the regression equation ( $F$ -to-enter = 34.1) and it was actually a *better* discriminator after accounting for covariation with the number of desmin-negative fibres ( $F$ -to-enter = 18.8 in Step no. 1 of Table 3). With both number of desmin-negative fibres and *Arpp/Ankrd2* expression levels, 91% of the experimental variability was explained. Interestingly, after accounting for correlation



**Figure 5. Expression levels of various mechanically sensitive genes 24 h after treatment**

A, CARP expression levels, B, *Arpp/Ankrd2* expression levels, C, MLP expression levels. Symbols at the top of the figure indicate significant differences between leftmost group (with arrowhead) and another group when connected with a vertical bar ( $P < 0.05$ ). Error bars are S.E.M.

**Table 3. Step 1 of stepwise regression analysis**

Parameter measured	Partial correlation ( <i>r</i> )	<i>F</i> -to-enter
Desmin-negative fibres	0.86	46.7*
Vimentin-positive fibres	0.81	33.0*
Fibronectin-positive fibres	0.85	44.2*
Embryonic MHC-positive fibres	0.42	3.6
Torque-time integral	0.58	8.6*
Pre-treatment torque	0.23	0.913
Peak torque	0.79	28.3*
Percentage torque drop	0.75	22.1*
<i>MyoD</i> mRNA	0.31	1.9
<i>MLP</i> mRNA	0.72	18.4*
<i>CARP</i> mRNA	0.81	32.8*
<i>Arpp/Ankrd2</i> mRNA	0.73	18.8*
<i>Embryonic MHC</i> mRNA	0.35	2.5

\*Values exceed the *F*-to-enter value of 4.000. For each step of the analysis, the *F*-to-enter value is an indicator of the extent to which the selected parameter is a good predictor of myogenin expression level. The correlation coefficient (*r*), quantifies the association between the selected parameter and the myogenin expression level.

**Table 4. Step 2 of stepwise regression analysis**

Parameter measured	Partial correlation ( <i>r</i> )	<i>F</i> -to-enter*
Vimentin-positive fibres	-0.12	0.22
Fibronectin-positive fibres	0.024	0.009
Embryonic MHC-positive fibres	0.21	0.77
Torque-time integral	0.10	0.16
Pre-treatment torque	-0.09	0.14
Peak torque	0.35	2.2
Percentage torque drop	0.42	3.4
<i>MyoD</i> mRNA	-0.02	0.01
<i>MLP</i> mRNA	0.33	1.9
<i>CARP</i> mRNA	0.57	7.7*
<i>Arpp/Ankrd2</i> mRNA	0.83	34.1*
<i>Embryonic MHC</i> mRNA	0.22	0.81

\*Values exceed the *F*-to-enter value of 4.000.

with number of desmin-negative fibres and *Arpp/Ankrd2* expression levels, *myoD* expression level then became a significant covariate with an *F*-to-enter of 5.3, resulting in a slightly higher value of  $r^2$  of 0.94 (Table 5). Thus, myogenin expression level was multifactorial and related to protein and transcript level alterations within the tissue. The crux of this multivariate analysis is that loss of desmin immunostaining was the best predictor of *myogenin* expression and was highly correlated with a number of other variables measured. *Arpp/Ankrd2* and *myoD* gene expression represented independent factors that uniquely affected *myogenin* gene expression to a lesser extent.

## Discussion

The purpose of this study was to define the effects of mechanical factors on gene expression in skeletal

**Table 5. Step 3 of stepwise regression analysis**

Parameter measured	Partial correlation ( <i>r</i> )	<i>F</i> -to-enter*
Vimentin-positive fibres	-0.25	1.0
Fibronectin-positive fibres	0.36	2.2
Embryonic MHC-positive fibres	0.11	0.21
Torque-time integral	0.25	0.96
Pre-treatment torque	-0.19	0.56
Peak torque	0.13	0.26
Percentage torque drop	0.17	0.43
<i>MyoD</i> mRNA	-0.51	5.3*
<i>MLP</i> mRNA	-0.36	2.3
<i>CARP</i> mRNA	0.03	0.01
<i>Embryonic MHC</i> mRNA	0.39	2.7

\*Value exceeds the *F*-to-enter value of 4.000.

muscle. We tested the hypothesis that eccentric contractions and isometric contractions (i.e. contraction mode), when performed at the same stress, yield unique results in muscle tissue. We found no unique effects of contraction mode when measuring either physiological or immunohistochemical parameters (Fig. 3, Table 2). However, gene expression levels were uniquely responsive to both contraction mode and treatment stress. Specifically, the *Arpp/Ankrd2* gene was sensitive only to contraction mode, independent of stress, while other genes measured responded to both stress and contraction mode.

Because we measured mechanical variables and gene expression in the same animal subjects, it was possible to define such interrelationships. The most interesting expression patterns was observed for *Arpp/Ankrd2* (Fig. 5B). This gene showed a clear dichotomous pattern – high expression levels for eccentric contractions, and low expression levels for isometric contractions. For the eccentric groups, peak dorsiflexion torque varied over the range 0.05–0.09 N m while for the isometric groups, peak torque varied over the range 0.03–0.06 N m. In spite of this wide variation, eccentric groups expressed *Arpp/Ankrd2* at a level almost three times that of each of the isometric groups (Fig. 5B). The eccentric contraction data obtained here are nearly identical to *Arpp/Ankrd2* expression levels ( $2.97 \pm 0.25$  fg ( $\mu\text{g RNA}^{-1}$ ),  $n = 5$ ) measured in a similar animal model 24 h after being stimulated at 100 Hz with the same eccentric contraction protocol (Peters *et al.* 2003). Importantly, the *Arpp/Ankrd2* gene has been implicated in mechanical signalling within muscle. For example, it is highly expressed in skeletal muscle and its expression is increased with denervation (Tsukamoto *et al.* 2002), chronic immobilization in a stretched position (Kemp *et al.* 2000), and strain in culture (Miller *et al.* 2003). Its intracellular distribution reveals a specific location in both the nucleus and the I-band (Tsukamoto *et al.* 2002) where it binds the amino terminus of the nebulin-anchoring

protein myopalladin (Bang *et al.* 2001) and possibly the N2A region of titin (Miller *et al.* 2003), and is thus strategically located to sense muscle cell strain. In a recent study of EC in a mouse model, we showed that *Arpp/Ankrd2* was rapidly upregulated within 12 h and returned to control levels within 48 h (Barash *et al.* 2004). Thus, at least three conditions have now been identified in which *Arpp/Ankrd2* expression is altered by changes in mechanical stress: strain in culture, chronic immobilization and eccentric exercise *in vivo*. However, this is the first demonstration that *Arpp/Ankrd2* expression might also be sensitive to the mode of contraction imposed upon the muscle. This field is in its infancy, only describing conditions under which *Arpp/Ankrd2* expression levels change. Subsequent mechanistic studies are under way to provide more detailed explanations for these observations. In order for *Arpp/Ankrd2* to be sensitive to active strain, it would have to be uniquely situated within the sarcomere to sense strain independent of stress. This might suggest a parallel connection to the titin molecule independent of the serial stress within the protein itself, perhaps mediated through *Arpp/Ankrd2*'s binding of myopalladin (Bang *et al.* 2001) or titin N2A (Miller *et al.* 2003), although the specific binding domains that could result in this configuration remain unknown. Another possible structural basis for the observed result would be a unique association between *Arpp/Ankrd2* and strain-activated ion channels, although there is as yet no evidence suggesting this.

In contrast to *Arpp/Ankrd2* expression, *myogenin*, *CARP* and *MLP* expression levels were highly correlated with peak torque and mode of muscle contraction. Correlations between the expression levels of these genes and peak torque varied from 0.73 to 0.78. The fact that not all of these genes entered the multiple regression model suggests that they might be coregulated since they were all highly correlated with one another (paired  $r^2$  values ranging from 0.81 to 0.89). Taken together, our interpretation of these data is that *myogenin*, *CARP* and *MLP* are sensitive to both muscle tissue stress and contraction mode, while *Arpp/Ankrd2* expression is sensitive only to contraction mode. A physical location that might enable proteins to sense stress would be the Z-disk, a structure that has long been thought to be involved in force transduction within skeletal muscle (Fridén *et al.* 1983). The recent study examining the numerous important Z-disk-associated proteins (Bang *et al.* 2001; Knöll *et al.* 2002) underscores its importance in transducing muscle signals. Of course one caveat to this discussion is that we have made the implicit assumption that the gene expression patterns measured reflect muscle cell expression rather than that of satellite cells, fibroblasts or blood cells. While this is the most likely interpretation, the use of immunohistochemistry and *in situ* hybridization are needed to confirm this assumption.

It should be noted that while this discussion is presented in terms of tissue stress, only dorsiflexion torque was directly measured. In this study we believe that it is reasonable to use dorsiflexion torque as a surrogate indicator of muscle stress. This is because no size differences were observed between animals, and therefore moment arms and physiological cross-sectional areas (PCSAs) were presumably identical so that torque would scale linearly with muscle stress. Based on the moment arms and PCSAs previously reported for this model (Peters *et al.* 2003), peak muscle stresses would be in the range of 200–350 kPa. These values would be at the high end of the physiological stress range, but certainly within the reported capability of rat skeletal muscle (Close, 1969).

We used peak torque measured 24 h after treatment as the main indicator of 'physiological injury.' This is due, in part, to the observation that some of these models showed partial recovery 24 h after treatment, suggesting that some of the initial torque decrease immediately after the exercise bout probably represented some type of neuromuscular fatigue. Peak torque was clearly the best predictor of physiological injury ( $P < 0.0001$ ,  $r^2 = 0.68$ ) as revealed by the multivariate analysis. This is consistent with a previous study of the rat soleus in which peak muscle force and injury were highly correlated (Warren *et al.* 1993). No indicator of any mode-specific cause of injury was seen, and there was no significant correlation between torque–time integral and torque measured 24 h after treatment. The fact that all immunohistochemical indicators of injury were significantly correlated with the peak torque and drop in torque implies that all of these parameters are indicators, albeit indirect, of muscle injury. It must be emphasized that, because the relationship between dorsiflexion torque and these parameters is completely asynchronous (Peters *et al.* 2003), this indicates that they are not causally related.

In conclusion, by varying the peak stress and the contraction mode imposed upon rat dorsiflexors, we demonstrated that peak muscle stress is the best predictor of muscle injury *in vivo*. When peak stresses were matched, no physiological or immunohistochemical differences were detected between isometric and eccentric contractions. This was contrasted greatly by the unique patterns of mRNA expression levels, which, in the case of *Arpp/Ankrd2*, was upregulated only with eccentric contractions but not by isometric contractions. The role that *Arpp/Ankrd2* upregulation plays in the biological response to eccentric contraction remains to be determined. Similarly, further study is required to understand the control mechanisms governing its expression as modulated by contraction mode, in contrast to certain other genes (such as *myoD*, *myogenin*, *MLP*, and *CARP*) which demonstrate sensitivity to tissue stress.

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