

Rapid muscle-specific gene expression changes after a single bout of eccentric contractions in the mouse

Iiona A. Barash, Liby Mathew, Allen F. Ryan, Ju Chen, and Richard L. Lieber

Departments of Orthopaedics, Bioengineering, Surgery, and Medicine and the Biomedical Sciences Graduate Group, University of California and Department of Veterans Affairs Medical Centers, San Diego, California 92161

Submitted 21 May 2003; accepted in final form 9 October 2003

Barash, Iiona A., Liby Mathew, Allen F. Ryan, Ju Chen, and Richard L. Lieber. Rapid muscle-specific gene expression changes after a single bout of eccentric contractions in the mouse. *Am J Physiol Cell Physiol* 286: C355–C364, 2004. First published October 15, 2003; 10.1152/ajpcell.00211.2003.—Eccentric contractions (ECs), in which a muscle is forced to lengthen while activated, result in muscle injury and, eventually, muscle strengthening and prevention of further injury. Although the mechanical basis of EC-induced injury has been studied in detail, the biological response of muscle is less well characterized. This study presents the development of a minimally invasive model of EC injury in the mouse, follows the time course of torque recovery after an injurious bout of ECs, and uses Affymetrix microarrays to compare the gene expression profile 48 h after ECs to both isometrically stimulated muscles and contralateral muscles. Torque dropped by ~55% immediately after the exercise bout and recovered to initial levels 7 days later. Thirty-six known genes were upregulated after ECs compared with contralateral and isometrically stimulated muscles, including five muscle-specific genes: muscle LIM protein (MLP), muscle ankyrin repeat proteins (MARF1 and -2; also known as cardiac ankyrin repeat protein and Arpp/Ankrd2, respectively), Xin, and myosin binding protein H. The time courses of MLP and MARF expression after the injury bout (determined by quantitative real-time polymerase chain reaction) indicate that these genes are rapidly induced, reaching a peak expression level of 6–11 times contralateral values 12–24 h after the EC bout and returning to baseline within 72 h. Very little gene induction was seen after either isometric activation or passive stretch, indicating that the MLP and MARF genes may play an important and specific role in the biological response of muscle to EC-induced injury.

muscle LIM protein; cardiac ankyrin repeat protein; muscle ankyrin repeat protein; microarray

THE BIOLOGICAL RESPONSE of skeletal muscle to eccentric contractions (ECs) remains poorly defined, despite numerous studies that have examined the structural and mechanical changes that occur. Documented structural changes include myofibrillar disruption and Z-line streaming (29), disruption of the cell membrane (28), and loss of immunostaining of the muscle-specific intermediate filament protein desmin (54, 55). In addition, there is evidence of increased nonuniformity of sarcomere length after ECs (79), disruption of the excitation-contraction coupling apparatus (85), and a severe drop in the maximum tetanic tension generated by the muscle (12, 41, 86).

These structural changes are likely to influence gene expression profiles, as profound changes occur in skeletal muscle specifically after ECs (as opposed to either isometric or concentric contractions) that eventually lead to muscle strength-

ening and protection of muscle against further EC-induced injury (61, 72, 75), although some studies have refuted this (42, 45). Understanding the mechanism of the injury and subsequent recovery from ECs may ultimately lead to more rational design of exercise regimens that minimize the pain and muscle injury required for strengthening. Furthermore, it may increase our understanding of the multiple factors that are involved with mechanical sensing in muscle.

Much work has focused on early signal transduction that occurs in muscle after exercise. Phosphorylation of members of the MAP kinase family occurs rapidly in both isolated (36, 71, 89) and in situ (5, 57) muscle preparations and also in response to exercise in humans (13, 47). Some studies have reported activation of these pathways even in response to passive stretch at high strains (14, 89), although this has only been demonstrated in vitro. Another signaling pathway implicated in the response of skeletal muscle to exercise is that involving IGF-I, the serine/threonine kinase Akt, the mammalian target of rapamycin (mTOR), and the translation activator p70^{s6k} (reviewed in Ref. 31). These signaling pathways, however, are ubiquitous to most tissues and are likely both downstream of sensors that respond to the initial stress or strain within muscle and upstream of muscle-specific adaptations to the exercise. A more complete explanation of the changes in muscle cells occurring with exercise, therefore, requires the identification of initial sensors within the muscle that can provide a link between the mechanical stimulus, cellular response, and structural elements that adapt to the stimulus.

One of the most intriguing potential stress sensors in muscle is the giant protein titin (mol wt 3,000,000–3,700,000), which connects the myosin thick filaments to the Z line (34, 49) and is a major contributing factor to the passive elastic properties of striated muscle (37, 49, 81). In addition, titin contains a number of unique domains that may participate in mechanotransduction. For example, the COOH terminus of the titin molecule is integrated in the myosin lattice and contains a serine/threonine kinase domain whose absence leads to sarcomeric disassembly (33). The Z line domain interacts with telethonin/T-cap (35), which in turn interacts with the muscle growth factor myostatin (64) and muscle LIM protein (44). Even the elastic segments found in the I-band (the N2A and N2B segments) are thought to be important in stretch-dependent sensing, interacting with such proteins as $\alpha\beta$ -crystallin, protein kinase A, and the muscle ankyrin repeat protein 1 [MARF1, also known as cardiac ankyrin repeat protein (CARP)] (10, 32, 90). Whether titin or the proteins that interact with titin play a role in the

Address for reprint requests and other correspondence: R. L. Lieber, Dept. of Orthopaedics (9151), VA Medical Center and Univ. of California-San Diego, 3350 La Jolla Village Dr., San Diego, CA 92161 (E-mail: rlieber@ucsd.edu).

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response of muscle to ECs remains to be determined. Any upregulation of the titin-interacting genes after ECs could indicate a significant role in EC-induced muscle remodeling. Another structural protein that seems to play an important role in the response to ECs is the muscle-specific intermediate filament protein desmin (51, 63). The extremely rapid mobilization of desmin after ECs (54, 55) and the fact that desmin knockout mice generate lower stress compared with their wild-type controls and yet exhibit less injury after ECs (73) suggest that desmin may play a key role in transmitting stress along the muscle cell and even in modulating muscle injury. The fact that the sarcomeric structure of the desmin-null mice shows greater variability and mobility (77) further suggests that desmin may play a key role in stabilizing the sarcomere and in stress transmission. Recent evidence showing increased desmin after ECs suggests that desmin regulation may also be involved with the adaptation of muscle fibers to ECs (12). We have reported (67) that desmin expression is increased after ECs in a rat model, with levels peaking 48 h after the EC bout. This suggests that muscle-specific cytoskeletal remodeling could play a role in the muscle's adaptation to ECs.

Despite the obvious potential roles of desmin, titin, and their associated proteins in the response of muscle to ECs, it is possible that some other, as yet unidentified, protein plays an important role. Microarray technology permits the probing of thousands of genes for differential expression between treatment groups, providing unbiased exploration across a huge range of genes and gene families. There have been relatively few microarray analyses examining gene expression changes after exercise in muscle. Those that have been performed have used models that are not specific to EC. For example, Booth and colleagues (19) examined gene expression differences between red and white skeletal muscle, Reecy and colleagues (20) looked at gene induction after synergist ablation-induced functional overload, and Esser and colleagues (21) used microarray analysis to study the response of muscle to a nonspecific EC model that was confounded by a huge fatigue component. Thus these models provide little information regarding the specific response of muscle to EC independent of repetitive activation and/or fatigue.

The goals of these experiments, therefore, were to develop a specific model of EC-induced injury in mice and to use microarray-based expression profiling to identify genes that could be considered "major players" in the biological response to ECs, as evidenced by their dramatic upregulation. There are at least two distinct roles that they could play: they could function as mechanosensors and/or signal transducers that initiate the program required for the appropriate response and adaptation to ECs, or they could be genes that are involved with the tissue adaptation itself. We chose to investigate the time period 48 h after an EC bout to emphasize this latter group, using desmin's expression time course (67) as the model. Ultimately, the information gained from these experiments will further our understanding of the response of skeletal muscle to exercise and how this response could be modified to increase muscle strengthening while limiting the extent of exercise-induced injury. A portion of these data has been presented in abstract form (11).

METHODS

Animals. Three- to four-month-old male 129/Sv mice (average weight 27.4 ± 0.279 g; Taconic Farms, Germantown, NY) were

housed four to a cage at 20–23°C with a 12:12-h dark-light cycle. All procedures were approved by the Department of Veterans Affairs (VA) Medical Center Committee on the Use of Animal Subjects in Research.

Experimental design. Mice underwent an exercise protocol of 50 isometric or eccentric contractions or 50 passive stretches, with maximal isometric torque recorded before and immediately after the exercise bout. At a specified time period (6, 12, 24, 48, 72, 120, or 168 h after the exercise bout), isometric torque was again measured, tibialis anterior (TA) muscles were removed, and the animals were euthanized. Only the 6, 12, and 48 h time points were studied in animals subjected to isometric contractions or passive stretches. Four animals were used at each time point for a total of fifty-six animals: twenty-eight animals were exercised eccentrically, twelve were subjected to isometric contractions, twelve were subjected to passive stretches, and four were used as controls and did not undergo any exercise protocol. Microarray analysis was performed by comparing the gene expression profiles of TA muscles 48 h after ECs to both TA muscles 48 h after isometric stimulation and to the contralateral TA muscles. Subsequently, quantitative real-time polymerase chain reaction (QPCR) was used to quantify the expression changes and define their time course after the exercise bout.

Exercise procedure. Under general anesthesia (2% isoflurane), mice were positioned in a specially designed jig that allowed measurement of ankle dorsiflexion torque as well as control over ankle position (to control muscle length) via an ergometer (custom-modified model 360B; Aurora Scientific, Ontario, Canada). Sterile subcutaneous 28-gauge needle electrodes (Grass Instruments, Braintree, MA) were placed in the vicinity of the right peroneal nerve, ~0.5 mm under the skin, just lateral to the midline and distal to the knee joint for stimulation of the TA and extensor digitorum longus (EDL) muscles. This experimental apparatus and stimulation procedure are similar to those used by Faulkner and colleagues (6). Proper electrode position was determined by a series of isometric single twitches over a range from 5 to 12 V; if the needle was not in the correct position, as the voltage increased the twitch amplitude decreased (and possibly became negative) as opposing muscles (soleus and gastrocnemius) were activated. The final stimulation voltage was determined by the peak twitch amplitude; if there was a plateau with no obvious peak, 7 V (corresponding to a current of ~25 mA) was used. Optimum stimulation frequency was determined by a series of three isometric contractions ranging from 150 to 250 Hz. The frequency resulting in a flat torque record was used for all subsequent contractions (usually 200 Hz). Preliminary experiments showed this to be on the plateau of the torque-frequency curve (data not shown), consistent with the results seen by others in mice (6, 16, 45, 87).

Before all exercise paradigms, maximal isometric torque was defined as the mean of two isometric contractions. During the stimulation protocols (eccentric and isometric groups only), the peroneal nerve was stimulated 50 times for 400 ms once per minute, with the footplate either remaining stationary (isometric group) or rotating 78° as the foot plantarflexed at ~1 fiber length/s (18) starting 150 ms after the nerve stimulation commenced (EC group). The starting tibiotarsal angle was ~60°, and the ending angle was ~140° (full plantarflexion), corresponding to a fiber strain of ~16%. As the normal active tibiotarsal angle ranges from ~50° to 80°, corresponding to sarcomere lengths of 2.2–2.4 μm in the mouse TA (52), it is likely that the majority of the EC occurred on the descending limb of the length-tension curve, with a maximum sarcomere length of ~2.9 μm (52). During the EC bout, isometric twitches were recorded every five contractions and the needle electrodes were repositioned if necessary to achieve a maximal twitch. Passive stretches were imposed on the muscle by rotating the footplate as described for the EC group, but without stimulation of the peroneal nerve.

The contralateral leg received only enough isometric contractions (~5) to determine and record maximum isometric torque, without ECs or passive stretches. The electrodes were then removed, and the

mice were returned to their cages. After the defined time interval (see *Experimental design*), isometric torque was again determined, TA muscles were removed and frozen in liquid nitrogen-cooled isopentane for further analysis, and the animals were euthanized by cervical dislocation while under anesthesia.

Preliminary data indicated that the stimulation protocol was repeatable, with no significant change in torque values when measured 24 h apart among seven animals ($P > 0.10$; data not shown). There was also no significant drop in torque during 50 isometric contractions administered 1 min apart ($P > 0.25$; data not shown), indicating that any drop in torque seen after ECs was not due to metabolic factors but rather was specific to EC-induced muscle injury.

RNA extraction/microarray analysis. Affymetrix microarrays (MGU74Av2; Affymetrix, Santa Clara, CA) were used to compare gene expression profiles 48 h after the isometric contraction and EC bouts and in contralateral limbs. After TA collection, RNA was extracted by using a combination of the standard Trizol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) protocols. In brief, 30 mg of frozen muscle was homogenized in a rotor-stator homogenizer on ice in 0.5 ml of Trizol; 0.1 ml of chloroform was added to the solution, which was then vigorously vortexed for 15 s followed by centrifugation at 4°C for 15 min. The upper aqueous layer was removed and mixed with an equal volume of 70% ethanol before being added to the RNeasy spin column. After the column was washed, it was incubated with RNase-free DNase (Qiagen) for 15 min and then washed again three more times before being eluted as described in the manufacturer's protocol. RNA concentration was determined by the absorbance at 260 nm, and the 260 nm-to-280 nm absorbance ratio was calculated to define RNA quality.

The goal of the microarray analysis was to identify muscle-specific genes that were highly upregulated after ECs. After the identity of these genes was determined, their expression levels were explored in more detail (other time points, other treatment paradigms) with QPCR. As a result, an analysis strategy was chosen that minimized the number of chips used, and therefore the sensitivity of the results, while only identifying genes that were dramatically differentially regulated. This procedure, termed the "twofold survival" method, was introduced (22) and then validated (7) in muscle by Hoffman and colleagues. Equal masses of RNA from four animals in each of three groups (eccentric exercise, isometric exercise, and contralateral muscles) 48 h after exercise were pooled and then hybridized onto two chips per group (denoted *A* and *B*) for a total of six chips. RNA processing for the microarray, including stringent quality control measures, was performed by the VA Gene Chip Core (Veterans Affairs San Diego Health Care System, San Diego, CA). GeneSpring software (SiliconGenetics, Redwood City, CA) was used to normalize the data both per chip (to the median of all elements on each chip) and per gene (to the median of that gene on all chips), and then genes were filtered such that only genes that were considered to be "present" by Microarray Suite 5.0 (Affymetrix) in at least two of the six chips were considered for further analysis.

Twofold survival analysis of the remaining genes was accomplished as follows. Each eccentric chip was compared in turn with each contralateral (*C*) chip, for a total of four comparisons (EC_A to C_A , EC_A to C_B , EC_B to C_A , EC_B to C_B). Genes that showed at least a twofold difference in every comparison were defined as differentially

regulated between eccentric and contralateral groups. An analogous comparison was then made between the eccentric chips and the isometric chips for four more comparisons testing for twofold expression differences. Genes that survived both sets of comparisons were defined as differentially regulated among EC, isometric contraction, and contralateral muscles, and these were the genes that were examined further.

QPCR. Quantification of RNA concentration was performed as follows. After RNA was extracted from the muscle as described in *RNA extraction/microarray analysis*, 500 ng from each sample was reverse transcribed with standard protocols (Superscript II; Invitrogen). cDNA was amplified with the Cepheid SmartCycler (Sunnyvale, CA) with primers specific to the genes of interest and then quantified by monitoring the fluorescence levels of the intercalating dye SYBRgreen (Sigma) compared with a PCR product standard. Primers for the muscle LIM protein (MLP) and MARP1 (CARP) were in the coding region, and primers for MARP2 (Ankrd2/Arpp) and MARP3 (DARP) were in the 3' untranslated region to insure specificity (Table 1). All primers were tested for cross-reactivity with other transcripts by using nBLAST and Oligo 6.6 (Molecular Biology Insights, Cascade, CO). All samples were run at least in duplicate, along with the standard. The PCR reaction vessel (25 μ l) contained 1 \times PCR buffer, 2 mM MgCl₂ (Invitrogen), 0.2 mM sense and antisense primers, 0.2 mM dNTP, 0.2 \times SYBRgreen, and 1 U of platinum *Taq* polymerase (Invitrogen). Amplification conditions were as follows. An initial hold at 95°C for 5 min was followed by 40 cycles of denaturing at 95°C for 15 s, followed by annealing/extension at 66°C for 40 s (MARP1), 70°C for 40 s (MARP2), or 69°C for 40 s (MLP and MARP3). The success of each reaction was deduced based on the observation of 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.

QPCR for MARP1, 2, and 3 and MLP was performed on RNA extracted from muscles collected 6, 12, 24, 48, 72, 120, and 168 h after the EC bout described above. Quantification of transcripts was also performed 6, 12, or 48 h after isometric contractions or passive stretch, in addition to eight control TA muscles from four animals not subjected to any treatment, to determine whether regulation of these genes was specific to ECs or whether regulation also occurred with passive stretch and isometric contractions as has been reported for other muscle models (43, 74, 88).

Statistical analysis. Experimental values from all animals in each treatment group at different time points were analyzed by two-way analysis of variance (ANOVA; Statview, SAS Institute) over time and group, and a one-way ANOVA was used to analyze each individual time between groups. In addition, a one-way ANOVA was used to analyze each experimental group (exercised and contralateral limbs) compared with the true control values. A repeated-measures design was not used, even though time was a dependent variable, because each time period was represented by different experimental subjects. Linear regression of each transcript against every other transcript was performed on QPCR data that were log₁₀ transformed before analysis to ensure a normal data distribution. Stepwise regression analysis of the expression levels of MLP, MARP1, MARP2, and MARP3 and their ability to predict torque loss was performed at each individual time point. $P < 0.05$ was considered to be significant, and all values are presented as means \pm SE.

Table 1. Primers used for quantitative real-time polymerase chain reaction

Gene	Accession No.	Sense Primer (5' - 3')	Antisense Primer (5' - 3')	Product Length, bp
MLP	D88791	ATCAGAGAAGTGCCACGATG	GTAAGCCCTCCAAACCCAAAT	213
MARP1	AF041847	GAAGTTAATGGAGGCTGGAGC	TTCTCTGTCCTTGGCATTGAG	244
MARP2	AJ011118	CGTGAGACTCAACCGTACAA	TGCAGGCAGCTCATAGTAGG	269
MARP3	AF492400	AACGCACAGGATAAGGAAGG	ATCCACAGGTGCCAGTCGTCA	228

MLP, muscle LIM protein; MARP, muscle ankyrin repeat protein.

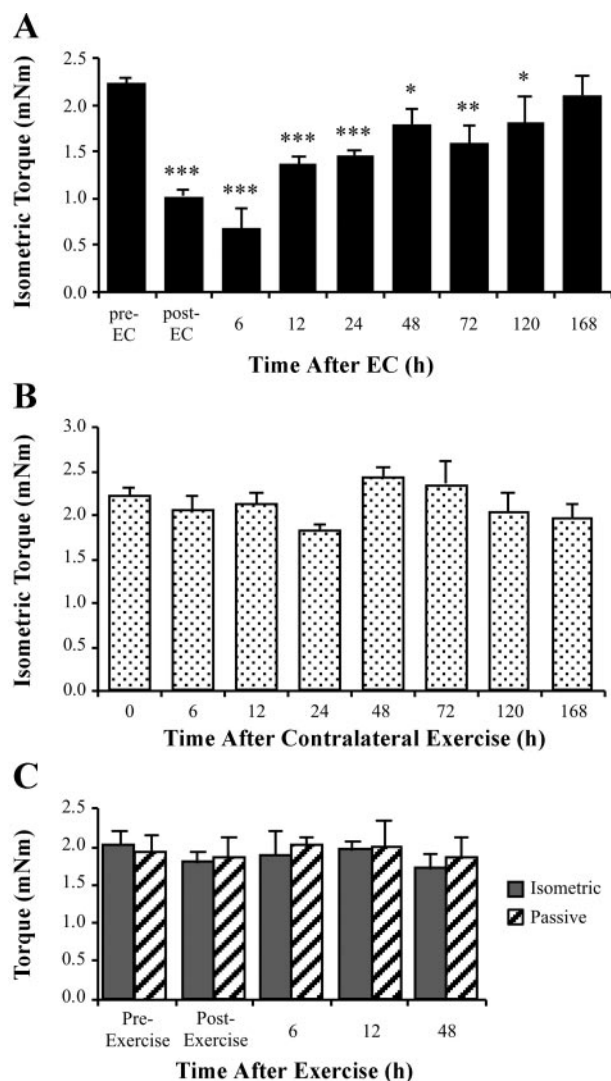


Fig. 1. Isometric torque loss after exercise. *A*: isometric torque before, immediately after, and 6, 12, 24, 48, 72, 120, and 168 h after a bout of 50 eccentric contractions (ECs). *** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$ compared with pre-EC torque. *B*: isometric torque of the leg contralateral to the eccentrically exercised leg immediately after and then 6, 12, 24, 48, 72, 120, and 168 h after the exercise. There is no significant change in torque at any time point. *C*: isometric torque loss before, immediately after, and 6, 12, and 48 h after a bout of either 50 isometric contractions or 50 passive stretches. There is no significant change in torque at any time point except pre- and postexercise values, which represent the combined initial torques for all animals in each group (28 for *A* and *B*; 12 for *C*).

RESULTS

Torque measurements. Two-way ANOVA of torque over time and between the eccentrically exercised muscle and contralateral muscle groups indicated a highly significant effect of time, group, and interaction between time and group ($P < 0.0001$ in all cases), highlighting the mechanically unique effect of ECs on mouse muscle. ECs resulted in an ~55% drop in torque immediately after the bout. This value decreased slightly more 6 h after the bout and then increased to the original values by 168 h after the bout (Fig. 1*A*). The contralateral leg, however, showed no significant change in torque over time (Fig. 1*B*). There was also no significant drop in torque immediately after or at any time after either isometric or

passive exercise bouts (Fig. 1*C*). These data are consistent with the injury seen in this model being specific for ECs and not a result of muscle activation (isometric muscles), muscle stretching (passive stretch muscles), or general malaise stemming from the anesthesia and procedure (contralateral muscles) and is also consistent with the literature (12, 17, 24, 62).

Microarray. Analysis of the microarray data by twofold survival analysis revealed 36 genes and 7 expressed sequence tags (ESTs) that were upregulated 48 h after ECs compared with both contralateral muscles and muscles subjected to isometric contractions alone (Table 2). In addition, five genes were determined to be downregulated in response to ECs (Table 3). The data were also analyzed for differential regulation between EC, isometric, and contralateral groups by Significance Analysis of Microarray (SAM) (83) and CyberT (9), with similar results obtained. These data have been deposited in GEO under accession number GSE435 (<http://www.ncbi.nlm.nih.gov/geo/>). It should be noted that the array used (Affymetrix U74Av2) does not contain any probes for titin, and therefore any changes in the expression level of this giant protein would not have been detected.

Table 2. Genes upregulated 48 h after an EC bout

Fold Change	Common Name	Accession No.
<i>Muscle-specific genes</i>		
8.08	Muscle Ankyrin Repeat Protein 1 (CARP)	AF041847
5.65	Muscle LIM Protein	D88791
4.66	Myosin Binding Protein H	U68267
4.65	Xin Protein	AF051945
3.98	Muscle Ankyrin Repeat Protein 2 (Ankrd2/Arpp)	AJ011118
<i>Non-muscle-specific genes</i>		
18.09	CD 53	X97227
15.52	LRG-21	U19118
14.13	A Disintegrin and Metalloprotease Domain (ADAM) 8	X13335
10.24	Small Inducible Cytokine B subfamily, member 5	U27267
7.74	EST	ZW214298
5.82	Arginase	U51805
5.36	EST	AW12227
5.21	Matrix Metalloproteinase 3	X66402
4.83	Monocyte Chemoattractant Protein-1 (MCP-1) receptor	U56819
4.44	Tx01	Z31362
4.36	Properdin factor, complement	X12905
4.31	Serum Amyloid A 3	X03505
4.27	Heat Shock Protein, 70 kDa	M12571
4.14	Small Inducible Cytokine A9	U49513
4.00	EST	A1504305
3.92	EST	AW061260
3.74	BIT (SHPS-1, SIRP α , P84)	AB01894
3.69	TYRO protein tyrosine kinase binding protein	AF024637
3.62	Ecotropic viral integration site 2	M34896
3.43	Heat Shock Protein 86	J04633
3.40	β Fc Receptor type II (FCRII)	M31312
3.33	Histocompatibility 2, class II antigen A, alpha	X52643
3.28	Intracellular calcium-binding protein, MRP14	M83219
3.13	EST	A1850362
3.09	Paired-Ig-like Receptor A1	U96682
3.08	Cathepsin S	AJ223208
2.93	α -2- β Crystallin	AV013428
2.79	EST	AW061237
2.76	Transforming Growth Factor, β induced (TGF- β)	L19932
2.73	Intracellular calcium-binding protein, MRP8	M83218
2.47	CD82	D14883

EC, eccentric contraction; EST, expressed sequence tag.

Table 3. *Genes downregulated 48 h after an EC bout*

Fold Change	Common Name	Accession No.
0.23	α-Actin, cardiac	M15501
0.27	Aquaporin 4	U88623
0.39	Pentylentetrazol-related PTZ-17	D45203
0.41	Integral membrane protein 2 (E25)	L38971
0.43	Glycerol-3-Phosphate Dehydrogenase (GPDH)	M25558

QPCR. Further analysis and confirmation of the microarray data by QPCR was performed for three of the muscle-specific genes: MLP and MARP1 and 2 (also known as CARP and Arpp or Ankrd2, respectively). A third recently identified member of the MARP family that was not on the Affymetrix chip, MARP3 (originally called DARP), was also analyzed (38).

Transcript analysis by QPCR of MLP, MARP1, and MARP2 showed a rapid increase in transcript levels peaking between 12

and 24 h after the exercise bout (Fig. 2, A–C). MLP expression levels showed a significant effect of time ($P < 0.0005$) and treatment ($P < 0.0001$) and a significant time \times treatment interaction ($P < 0.0005$; Fig. 2A), peaking 24 h after the EC bout at 10 times higher than contralateral muscles. MARP1 showed varied expression levels even in the contralateral leg, but two-way ANOVA showed a highly significant effect of time and treatment and a highly significant time \times treatment interaction on expression level ($P < 0.0001$ in all cases; Fig. 2B). Peak MARP1 levels 12 h after injury were ninefold higher than the contralateral values. MARP2 showed a similar expression pattern, although at much lower relative levels compared with MARP1 ($P < 0.0001$ in all cases; Fig. 2C), peaking 12 h after injury at a level sixfold higher than contralateral values. The transcript levels of all three genes returned to baseline levels quickly: MLP and MARP2 within 48 h and MARP1 within 24 h. The ratios between transcript levels of the exercised vs. contralateral muscles, however, were still high 48 h

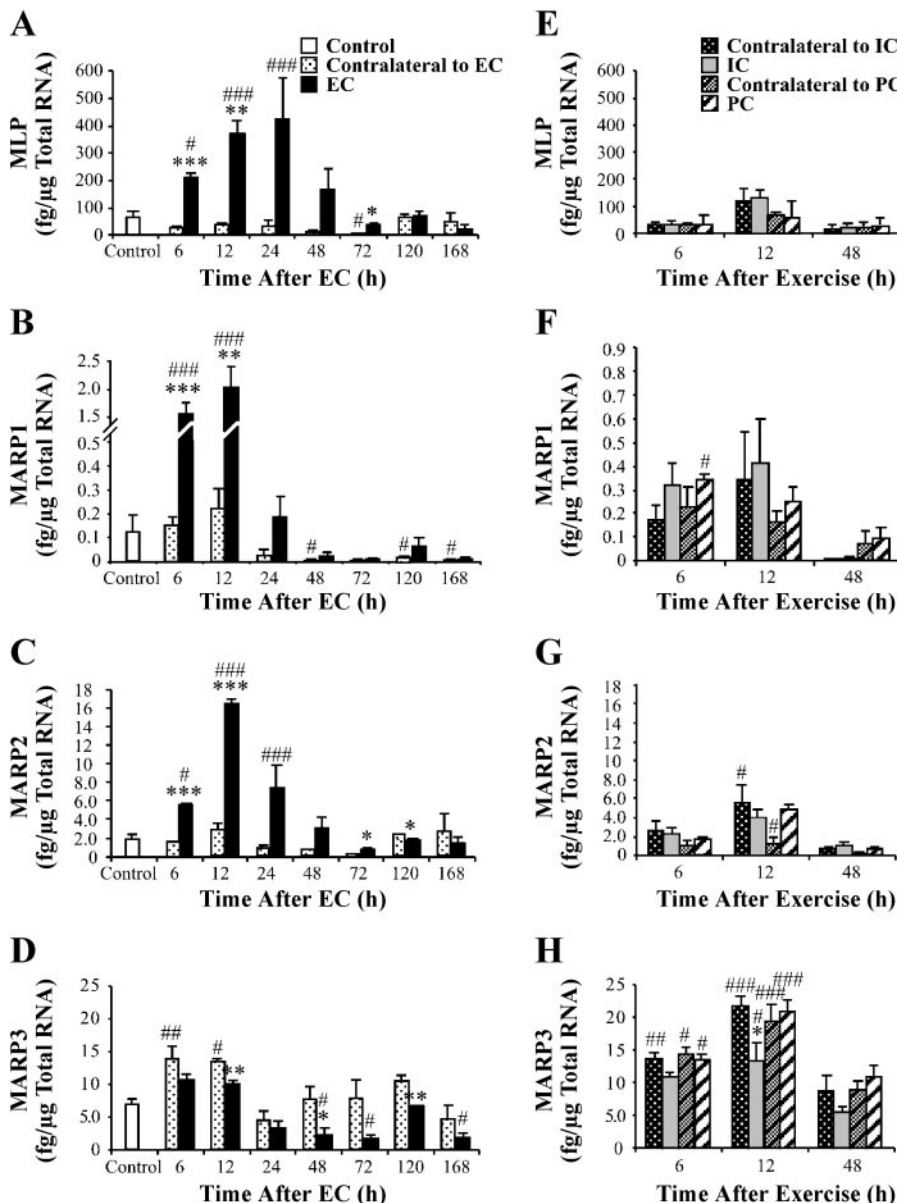


Fig. 2. Quantitative PCR mRNA measurements of muscle LIM protein (MLP) and muscle ankyrin repeat protein (MARP)1, MARP2, and MARP3 after exercise. A–D: mRNA concentrations after EC along with values for the muscles contralateral to the EC muscles and control muscles, which were not subjected to any exercise protocol. E–H: mRNA concentrations after either isometric (IC) or passive exercise (PC), along with their contralateral muscles. A and E: values for MLP. B and F: values for MARP1. C and G: values for MARP2. D and H: values for MARP3. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$ compared with contralateral concentrations; ### $P < 0.0001$, ## $P < 0.001$, # $P < 0.05$ compared with true control concentrations; $n = 4$ muscles in all cases except for control values, which represent 8 muscles from 4 animals.

Table 4. Comparison of fold change between eccentrically stimulated and contralateral muscles 48 h after EC bout as determined by microarray and QPCR

Gene	Fold Change	
	Microarray	QPCR
MLP	5.65	15.56
MARP1	8.08	4.05
MARP2	3.98	5.75

QPCR, quantitative real-time polymerase chain reaction.

after EC, consistent with the microarray analysis (Table 4). This confirms the results of the microarray analysis and provides further information regarding the temporal regulation of these genes.

Interestingly, contralateral muscles showed variable expression of these genes as well (Fig. 2). The most dramatic change was seen with MARP1, whose expression in the contralateral limb decreased 24 h after the EC and reaching statistical significance at 48 h (overall $P < 0.0001$; Fig. 2B), despite receiving only enough contractions to permit accurate recording of torque (~5 isometric contractions). Significant changes in contralateral limbs were also seen with MARP3, with increased expression 6 and 12 h after stimulation of the experimental limb ($P < 0.05$; Fig. 2D).

Compared with EC, transcript levels of these three proteins did not change as dramatically after either isometric contractions or passive stretch (Fig. 2, E–G). No significant differences between the isometric contraction and passive stretch groups and their respective contralateral limbs were detected, whereas minor differences were detected compared with the true control expression levels. This indicates that changes in expression of MLP, MARP1, and MARP2 are likely to be EC specific, possibly a result of the extremely high tensions and/or active strain borne by the muscle (53). Regression analysis indicated that mRNA levels of MARP1, MARP2, and MLP were significantly correlated with each other even though all changes were biphasic in nature and out of phase in time (Fig. 3), suggesting the possibility of common regulatory factors (see DISCUSSION).

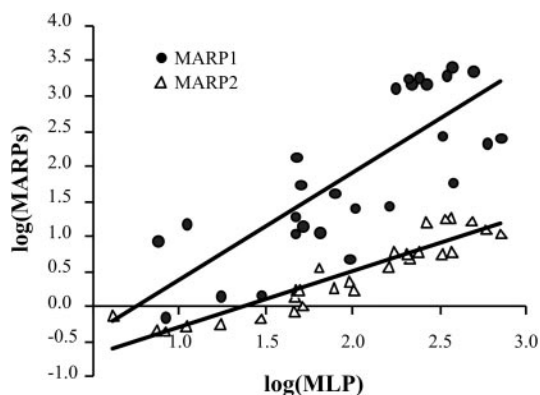


Fig. 3. Linear regression relationship between MLP and MARP1 and MARP2. All values were \log_{10} transformed before analysis, and only mRNA expression levels from the EC group were plotted. Both MARP1 and MARP2 mRNA levels are significantly related to the levels of MLP ($r^2 = 0.616$ and $r^2 = 0.891$, respectively; $P < 0.0001$ in both cases).

MARP3 transcript analysis revealed a less consistent time course. Perhaps as a result of the increase in expression of the other MARPs after ECs, there was a significant downregulation of MARP3 in the muscle exposed to EC compared with the contralateral muscles starting 12 h after the EC bout (Fig. 2D), suggesting that the three MARP family members may regulate or compensate for each other. MARP3 expression was not dramatically influenced by either isometric or passive contractions (although there was a slight decrease in expression levels after isometric stimulation; Fig. 1H) in the exercised limb compared with the contralateral limb. Compared with true controls, however, MARP3 expression was dramatically increased both 6 and 12 h after either isometric or passive exercise in both the stimulated and contralateral limbs. Whether this is a real effect of the exercise or an artifact of individual animal variability remains to be determined.

Stepwise regression of the initial torque loss as a function of the expression of MLP and the MARPs revealed that all four genes were influenced by the magnitude of the initial torque loss at various time periods after EC (Table 5)—MARP1 and MARP2 12 h after the EC bout, MARP1 and MARP3 48 h after the bout, and MLP 120 h after the exercise—predicting the initial torque loss to within 28% of its actual value. The fact that two genes entered the stepwise regression model at 12 and 48 h indicates responses by these genes that are independent of one another. Additionally, because stepwise regression can be biased toward the parameter with the lowest coefficient of variation, we confirmed that, in all cases, the genes that entered the regression model were not necessarily those with the lowest coefficient of variation. For example, MARP1, which had a coefficient of variation of 114% at 48 h, entered the regression relationship at this time point whereas MARP2, which had a coefficient of variation of only 74%, did not.

DISCUSSION

This study presents the time course of recovery after EC-induced injury in a minimally invasive mouse model and examines the gene expression profile 48 h after the injury. Maximum isometric torque decreased 55% immediately after the bout of 50 ECs and was fully recovered 7 days later. This is consistent with results reported previously in an analogous rat model (12). It remains unknown whether the torque decrease measured immediately after the EC, and recovering gradually thereafter, is due to mechanical failure of the muscle (reviewed in Ref. 70), excitation-contraction uncoupling (reviewed in Ref. 85), or other unidentified factors.

Microarray analysis revealed 36 upregulated genes in the EC group compared with contralateral muscles and those subjected

Table 5. Stepwise regression analysis of initial torque loss after ECs as a function of expression levels of MLP, MARP1, MARP2, and MARP3

Time, h	Equation: $y(\% \text{pre-EC torque}) =$	R^2	P
12	$0.0001(\text{MARP1}) + 0.0635(\text{MARP2}) - 0.909$	1.000	0.007
48	$0.0009(\text{MARP1}) + 0.0688(\text{MARP3}) + 0.275$	0.999	0.015
120	$0.0052(\text{MLP}) + 0.175$	0.727	0.095

Significant stepwise regression relationships were only found for the time points shown. The units for all transcript levels are $\text{fg}/\mu\text{g}$ total RNA. %pre-EC torque is calculated as the isometric torque immediately after the 50 ECs divided by the torque before initiation of the EC bout.

only to isometric stimulation (Table 2). One upregulated gene (arginase) is a metabolic gene, catalyzing the conversion of arginine into ornithine and urea. The significance of this finding remains unclear. Twenty-four genes were not muscle specific, and many of these were likely upregulated as a result of the extensive inflammation and macrophage infiltration that occurs after EC injury (4, 50, 59, 68). One-quarter of these genes (LRG-21, ADAM8, small inducible cytokine A9, BIT, FCR2, and cathepsin S) are most likely associated with macrophage infiltration, whereas seven other genes are likely upregulated as a result of the infiltration of other immune cells, including T cells (CD53, MHC 2 class II antigen A), B cells (Paired Ig-like receptor A1), natural killer cells (TYRO binding protein), and neutrophils (MRP14, MRP8, CD82). Other categories of upregulated genes include those involved with extracellular matrix remodeling (MMP3) and inflammation (small inducible cytokine B5, serum amyloid A3). In addition, three heat shock proteins (HSP70, HSP86, α 2 β -crystallin) previously implicated in mediating the response of muscle to exercise and stress (26, 80) were upregulated, along with increased expression of TGF- β , likely resulting in angiogenesis (15). Interestingly, seven of the identified genes, including representatives from across the entire spectrum of “non-muscle-specific” genes (CD53, MMP3, properdin factor, small inducible cytokine A9, BIT, TYRO binding protein, and cathepsin S) have also been shown to be upregulated in the mouse model of Duchenne muscular dystrophy (DMD), the *mdx* mouse (69). Muscles of these mice exhibit a large degree of regeneration and inflammation, which may be due to mechanisms also operational in our EC model.

Importantly, five upregulated genes are predominantly found in muscle and are therefore likely to be involved with muscle-specific response to the exercise-induced injury. Xin protein is a recently identified gene thought to be important in cardiac development and is found in some skeletal muscle cell-cell junctions in adults (78, 84). Myosin binding protein H (MyBPH) binds myosin in the A band, may be involved with the stabilization or localization of myosin within the sarcomere (30, 76), and has been shown to be upregulated in muscles of patients with DMD (8). The three genes that we focused on were MLP, MARP1, and MARP2, because these genes have all been speculated to participate in mechanical signaling (10, 43, 44).

MLP was originally discovered as being upregulated after denervation of skeletal muscle (2) and has since been shown to be upregulated during fast-to-slow fiber type transitions (19, 74, 88). It has the intriguing attribute of being primarily cytoplasmic in adults but nuclear in embryos (2). While in the nucleus, it binds to and increases the activity of the myogenic regulatory factor MyoD (46). In the cytoplasm, it binds the structural proteins β -spectrin, telethonin, and α -actinin (27, 44) and has therefore been postulated to be a mechanical sensor in muscle cells. A knockout model of this mouse is available (3) in which adult mice possess severe cardiac hypertrophy, myofibrillar disarray, and less compliant hearts (25, 65). MLP has already been shown to be upregulated in a complex model of EC that includes high levels of fatigue (21). In our model, MLP levels peaked 24 h after the EC bout and then returned to baseline by \sim 72 h. Compared with the expression changes in the contralateral limb, MLP expression did not change with either passive stretch or isometric stimulation alone (Fig. 2E).

Others (74) have seen an increase in expression of MLP after isometric stimulation, and in fact in our model there is a slight increase in expression 12 h after isometric stimulation compared with unstimulated controls; however, this increase was also observed in the contralateral limb (Fig. 2E).

MARP1 is also known as cardiac ankyrin repeat protein or cardiac adriamycin responsive protein (CARP) or C-193 and was originally discovered as a cytokine-inducible gene in fibroblasts before being shown to be differentially regulated between embryonic and adult heart muscle (23, 40, 48, 91). We have chosen to call this gene, and its two related counterparts, muscle ankyrin repeat proteins (MARPs) to reflect their predominant expression in striated muscle and the high level of homology among the three proteins. MARP1 is thought to be a marker for cardiac hypertrophy (1, 48) and is also a transcription inhibitor that acts downstream of the homeobox gene *Nkx2.5* (91). Like MLP, MARP1 is located in both the nucleus and the cytoplasm (39, 91). MARP1, however, is found in the central I band of the sarcomere, where it binds the amino terminus of the nebulin anchoring protein myopalladin (10). Other studies have shown MARP1 upregulation in response to work-overload hypertrophy (20) and acute resistance exercise (21), and in muscle from patients with DMD (8). In our study, MARP1 expression was dramatically upregulated 6–12 h after ECs (9-fold compared with contralateral muscles) and returned to baseline 24 h later. This extremely rapid response of the MARP1 gene to exercise implicates it as one of the most responsive muscle genes ever identified in muscle adaptation—almost as fast as the immediate-early genes *c-fos* and *c-jun* (66).

MARP2 (also known as Arpp and Ankrd2) is similar to MARP1, although it is more highly expressed in skeletal than in cardiac muscle, preferentially in type 1 fibers (43, 82). Its expression in skeletal muscle is increased with denervation (82), chronic immobilization in a stretched position, and differentiation in culture (43). It seems to have the same intracellular distribution as MARP1, being located in the nucleus or the I band (82). In this study, MARP2 expression, like MARP1, increased dramatically after EC, peaking 12 h after the EC bout and returning to baseline within 24 h. We failed to see an increase in expression of this gene in response to passive stretch alone, in contrast to the result seen by others (43). This discrepancy may be due to the dramatically different stretch protocols used (immobilization in a stretched position for 7 days compared with 50 passive stretches over a 1-h period).

The strong regression relationship between MLP, MARP1, and MARP2 suggests that mRNA expression of these three genes may be regulated by similar processes (Fig. 3). We are unaware of any literature regarding the translational control or stability of MARPs or MLP mRNAs that might influence their expression patterns. A preliminary analysis of transcription factor binding sites 2 kb upstream of the MARP1, MARP2, and MLP genes using analysis tools found on VISTA (56, 58) revealed binding motifs for 65 transcription factors that were common to all three genes. Some of these motifs formed clusters suggestive of regulatory domains. Factors that may bind to these motifs are potential candidates for coregulation of the three genes. It is of course possible that other factors, or sites at other locations in the genes in question, could be responsible for coregulation. In the absence of experimental

data, the question of which if any of these factors are functionally important remains unanswered.

MARP1 and MARP2 are ~50% homologous (60, 82), and a third member of the MARP family (MARP3, also known as diabetes-associated repeat protein) has recently been cloned (38). Because it is newly identified, this gene was not represented on the microarray used for the initial analysis, but it is thought to be more prevalent in adult skeletal muscle than in cardiac muscle. Our data indicate that the regulation of MARP3 expression is entirely different than that of MARP1, MARP2, or MLP, such that MARP 3 expression, if anything, decreases after the EC bout, perhaps in response to increased MARP1 and MARP2. Interestingly, the expression of all four genes is related to the initial amount of injury seen in the muscle, shown by stepwise regression analysis of the initial torque decrement and transcript expression (Table 5). The importance of the specific times at which the expression of MLP, MARP1, MARP2, and MARP3 are related to the initial torque loss (12, 48, or 120 h) remains unclear. The fact that all four genes ultimately correlate with initial torque loss at some time point, however, indicates that all four genes are likely influenced by the magnitude of the initial EC-induced injury.

The time point chosen for microarray analysis (48 h after EC) was based on studies in our laboratory indicating that this was the peak of transcriptional activity for the muscle-specific intermediate filament protein desmin (as opposed to generic transcription factors and early response genes) after EC in a rat model. The initial goal of the experiments, therefore, was to determine whether other as yet unidentified muscle-specific cytoskeletal elements were upregulated at this point as well. It may well be that an examination of earlier time points would lead to a different list of less tissue-specific significant genes, as found by others (21). In addition, we chose to use the twofold survival method of microarray analysis, to identify genes that were most dramatically regulated after the EC bout. A disadvantage of this method is that it is heavily biased toward highly expressed transcripts and therefore is unlikely to identify smaller but still potentially significant changes in genes of lower copy number. The advantage of this method, however, is its stringency, resulting in a very low "false positive" identification rate for differentially regulated genes. Interestingly, when the same data were analyzed with SAM (83), the only other muscle-specific gene upregulated after EC was MyoD, indicating that the twofold survival analysis was sensitive in addition to being specific.

One other potentially confounding aspect of our approach is that all MARP1 expression levels, including contralateral muscles and muscles subjected to only isometric stimulation or passive stretch, are much lower 24–48 h after the initial exercise bout compared with earlier time points. Expression levels of "true control" muscles, which did not receive any exercise stimulation, are similar to those found 6–12 h after EC in the contralateral leg (Fig. 2). The interpretation of the subsequent drop in mRNA concentration 24 h after any stimulation, even in the contralateral leg, remains unclear but could potentially be attributable to individual differences between animals (note that the mRNA at each time point comes from a separate group of animals), the small amount of isometric activation of the contralateral limb needed to obtain accurate torque readings, a systemic effect of the exercise, a general stress response, or a rebound effect of increased expression at

early time points leading to reduced expression at later time points.

In conclusion, this experiment developed and validated a model for ECs in the mouse and completed microarray analysis of the TA muscle 48 h after the EC bout. The analysis uncovered three interesting muscle-specific genes (MARP1, MARP2, and MLP) whose time-dependent regulation was later confirmed by QPCR analysis to be specific to the EC treatment as opposed to either isometric activation of the muscle or passive stretch. The role of these genes in the biological response to ECs in skeletal muscle remains to be determined, but their rapid regulation indicates that the role is an important upstream event associated with muscle response to EC.

ACKNOWLEDGMENTS

We thank Dr. Marie-Louise Bang for helpful discussions. The sequences for the promoter analysis were generated through the use of the Celera Discovery System.

GRANTS

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-40050 and the Department of Veterans Affairs.

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