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ORIGINAL ARTICLE

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Differentially expressed genes and morphological changes during lengthened immobilization in rat soleus muscle

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Abstract To examine the effect of lengthened immobilization on the expression of genes and concomitant morphological changes in soleus muscle, rat hindlimbs were immobilized at the ankle in full dorsiflexion by plaster cast. After removing the muscle (after 1 hr, 1, 4, and 7 days of immobilization), morphology and differential gene expression were analyzed through electron microscopy and differential display reverse transcription-polymerase chain reaction (DDRT-PCR), respectively. At the myotendinous junction (MTJ), a large cytoplasmic space appeared after 1 hr of immobilization and became enlarged over time, together with damaged Z lines. Interfibrillar space was detected after 1 day of immobilization, but diminished after 7 days. At the muscle belly, Z-line streaming and widening were observed following 1 hr of immobilization. Disorganization of myofilaments (misalignment of adjacent sarcomeres, distortion, or absence of Z lines) was detected after 4 days. Furthermore, mitochondrial swelling and cristae disruption were observed after 1 day of stretching. A set of 15 differentially expressed candidate genes was identified through DDRT-PCR. Of 11 known genes, seven (*Atp5g3, TOM22, INrf2, Slc25a4, Hdac6, Tpm1*, and *Sv2b*) were up and three (*Podxl, Myh1,* and *Surf1*) were down-regulated following immobilization. In the case of *Acyp2*, 1-day stretching-specific expression was observed. Atp5g3, Slc25a4, TOM22, and Surf1 are mitochondrial proteins related to energy metabolism, except TOM22, which has a chaperone-like activity located in the mitochondrial outer membrane. Together with these, INrf2, Hdac6, Podxl, and Acyp2 are related more or less to stress-induced apoptosis, indicating the responses to apoptotic changes in mitochondria caused by stretching. The expression of both Tpm1 and Myh1, fast twitch isoforms, suggests adaption to the immobilization. These results altogether indicate that lengthened immobilization regulates the expression of several stress/apoptosis-related and muscle-specific genes responsible for the slow-to-fast transition in soleus muscle despite profound muscle atrophy.

Key words skeletal muscle · lengthened immobilization · differentially expressed genes · morphological changes

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Introduction

Muscle stretch is a general therapeutic maneuver used to increase range of motion (ROM) by elongating structures that have adaptively shortened and become hypomobile for a long time. Several studies have examined the effects of muscle stretch in a lengthened position (Tabary et al., 1972; Williams and Goldspink, 1973; Dix and Eisenberg, 1990; Williams, 1990), and suggested that the stretched muscle fibers increase the number of sarcomeres to maintain a normal passive

length–tension relationship (Caiozzo et al., 2002). For this reason, muscle stretch is often applied in clinical rehabilitation therapy. However, little is known about the molecular mechanisms occurring during this process.

In general, muscle stretch is sensed at the sarcolemma, most likely by a complex of integral membrane proteins, and signal transduction leads cytoplasmic responses such as the phosphorylation of transcription factor(s), translocation of these factors into the nucleus, specific gene (DNA) transcription, and translation into specific proteins (De Deyne, 2001). Using methods like Northern blotting, immunoblot, and RNA protection analysis, a number of genes, such as insulin-like growth factor-1 (*IGF-1*; McKoy et al., 1999), myosin heavy chain (*MHC*; Galler et al., 1997), dihydropyridine receptor (*DHPR*; Radzyukevich and Heiny, 2004), sarcoplasmic/endoplasmic reticulum Ca (2+)-ATPases (*SERCA1*; Zador et al., 1999), mammalian target of rapamycin (*mTOR*; Hornberger et al., 2004), myogenin (Ikeda et al., 2004), *c-fos* (Ikeda et al., 2003), and ankyrin repeat domain 2 (*Ankrd2*; Kojic et al., 2004), have been identified to be expressed during and/or after muscle stretch. Through differential screening, Cros et al. (2001) analyzed 34 differentially expressed genes in rat soleus muscle atrophied by hindlimb suspension. The serial analysis of gene expression (SAGE) method was also used to elucidate the molecular basis of muscle atrophy induced by a cast in a shortened position (St-Amand et al., 2001). They reported 47 genes, categorized into three groups according to their functions: contraction, energy metabolism, and housekeeping. Until now, however, systematic gene expression analysis has not been performed in the muscle following different periods of stretch time.

In the case of the immobilization of muscle in a shortened position, reduction of muscle fiber length has been reported due to a loss of sarcomeres. However, intermittent stretches of 1/2, 1, or 2 hr daily for a period of 2 weeks have been reported to increase the number of sarcomeres following immobilization in a shortened position (Williams, 1990). Electron microscopy (EM) studies have revealed a large cytoplasmic space containing polysomes, sarcoplasmic reticulum and T membranes, mitochondria, Golgi complexes, and nascent filament assemblies at the myotendinous junction (MTJ) of 4-day stretched muscle fiber. *In situ* hybridization analysis found a high level of MCH mRNA expression at the MTJ of stretched muscle fibers (Dix and Eisenberg, 1990). A novel stretch-responsive skeletal muscle gene (*Smpx*) was identified through expression on the muscle fiber after 7 days of passive stretching, and it may play a role during muscle hypertrophy (Kemp et al., 2001). Other studies have also reported an increase in sarcomere number after casting of hindlimbs in a lengthened position for 3, 4, or 1–7 weeks (Tabary et al., 1972; Williams and Goldspink, 1978).

Because previous studies have not examined the systematic gene expression pattern following different periods of stretch time, we applied a differential display reverse transcription-polymerase chain reaction (DDRT-PCR) in an attempt to analyze the differentially (up or down-regulated) expressed genes systematically according to different periods of muscle stretching (1 hr, 1, 4, and 7 days) as well as the concomitant morphological changes.

Material and methods

Animals

Thirty-five Sprague–Dawley male rats weighting 300–320 g were used. Both hindlimbs were immobilized at the ankle in full dorsiflexion (about 55°) by plaster casts for 1 hr, 1, 4, and 7 days except in the control group. Hindlimbs of 25 rats were used for EM studies and their left hindlimbs were taken out for DDRT-PCR. Ten rats were used *for in situ* hybridization. For DDRT-PCR and *in situ* hybridization, soleus muscles were rapidly excised, immediately frozen in liquid nitrogen, and stored at -80°C. For EM, the soleus muscles from each limb were isolated, their length were measured, and weighted.

EM

Small blocks of tissue (1 mm^3) from the mid-portion and MTJ portion of the muscle were immersed overnight at 4°C in 0.1 M phosphate buffer of pH 7.4 containing 2.5% glutaraldehyde, and then post-fixed with 1% osmium tetroxide for 1 hr. Tissue samples were longitudinally oriented and embedded in epoxy resin (Spurr's). Four sample blocks were obtained from each muscle sample. The sample blocks from each biopsy were trimmed of excess plastic resin and sectioned using a glass knife for thick sections and a diamond knife (DIATOME, Hatfield, PA) for thin sections. Thick sections (1 μm) were cut from the blocks and stained with toluidine blue (0.2% in 2.0% sodium borate) at 60°C for 1 min. Thin sections (90 nm) were then cut, stained with saturated uranyl acetate for 5 min and lead citrate for 3 min, and examined using a transmission electron microscope (JEM-1200 EX; JEOL, Tokyo, Japan).

RNA isolation

Total muscle RNA was isolated by the acid guanidium thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987). Briefly, soleus muscles were homogenized in 4 M guanidium isothiocyanate solution. The following solutions were added in succession with vortexing 2–5 min each time: 2 M sodium acetate pH 4, followed by water-saturated phenol, and then by chloroform-isoamyl alcohol. After centrifugation, the upper aqueous phase was taken and 2 vol of ethanol were added and kept overnight at -20°C. After centrifugation, the RNA pellet was washed with 75% ethanol and dried in air. The RNAs obtained were quantitated using a spectrophotometer Ultraspec 2000 (Pharmacia Biotech, Piscataway, NJ). The quality of the RNA was assessed by visual inspection of ethidium bromide-stained 18S and 28S rRNA under ultraviolet light.

DDRT-PCR

A GenHunter RNAimage® kit 1 (GenHunter Co., Nashville, TN) was used for DDRT-PCR analysis. After synthesizing the cDNA