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Increase of Myoglobin in Rat Gastrocnemius Muscles with Immobilization-induced Atrophy

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Abstract. [Purpose] Atrophy is a common phenomenon caused by prolonged muscle disuse associated with bed-rest, aging, and immobilization. However, changes in the expression of atrophy-related myoglobin are still poorly understood. In the present study, we examined whether or not myoglobin expression is altered in the gastrocnemius muscles of rats after seven days of cast immobilization. [Methods] We conducted a protein expression and high-resolution differential proteomic analysis using, two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry, and western blotting. [Results] The density and expression of myoglobin increased significantly more in atrophic gastrocnemius muscle strips than they did in the control group. [Conclusion] The results suggest that cast immobilization-induced atrophy may be related to changes in the expression of myoglobin in rat gastrocnemius muscles.
Key words: Myoglobin, Gastrocnemius muscle, Atrophy

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INTRODUCTION

Myoglobin is a cytoplasmic hemoprotein consisting of a single polypeptide chain of 154 amino acids. It is capable of binding a wide variety of ligands, such as dioxygen (O_2) and nitric oxide (NO)[1, 2)]. The major function of myoglobin as a dioxygen store is to supply dioxygen to peripherial tissues[1, 3)]. Furthermore, myoglobin may serve as an important scavenger of NO in the heart and skeletal muscles[4, 5)]. Myoglobin is also known to have peroxidase activity and is a scavenger of reactive oxygen species (ROS)[6–8)]. Although myoglobin is expressed at low levels in the hind-limb muscles of neonatal animals, as postnatal development proceeds and progressive locomotor activity increases, the quiescent expression level of myoglobin increases in the muscle fibers[9)]. Muscle atrophy has proven to be a significant problem in the field of orthopedic physical therapy within rehabilitation[10–12)]. All atrophic conditions are related as they involve a loss of protein resulting from impairment of metabolism[10–12)]. However, changes in the expression of myoglobin in skeletal muscle dysfunction, especially during immobilization-induced atrophic conditions, are not fully understood[12)]. Therefore, in the present study, we investigated the changes in the expression of myoglobin in the gastrocnemius muscles of rats during cast immobilization.

MATERIALS AND METHODS

Male Sprague-Dawley rats were housed in a temperature and humidity controlled room under a 12-hour light/dark cycle and fed a standard commercial chow *ad libitum*. The rats (n=25) were anaesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) mixed with xylazine hydrochloride (5 mg/kg) and a plaster of paris cast was attached[10)]. Experimental procedures on two-dimensional gel electrophoresis (2-DE) and mass spectrometry were as described in previous reports[12)]. Gastrocnemius muscle strips, from animals with or without cast immobilization for seven days, were lysed. The protein homogenates were diluted with rehydration buffer. The immobilized

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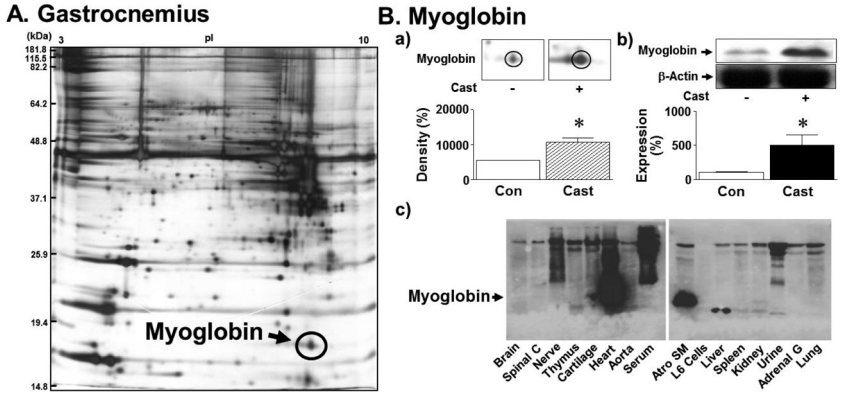

Fig. 1. Change of silver-stained 2-DE gels of myoglobin, and expression of myoglobin detected by immunoblotting of rat hind-limb gastrocnemius muscles after seven days of cast immobilization.
(A, B-a) Proteins were subjected to IEF on IPG pH 3-10 nonlinear strips and then separated by 12% (w/v) polyacrylamide SDS gels. Statistical analysis was performed on gels from four independent experiments using PDQuest. Arrows show myoglobin protein examined by MALDI-TOF/TOF. (B-b, B-c) Immunoblotting analysis of the cast-immobilized skeletal muscle. *, Significantly different from day 0 of non-cast control, $p < 0.05$. Con, control; Cast, cast immobilization; pI, isoelectric point; Spinal C, spinal cord; Atro SM, atrophied skeletal muscle; Adrenal G, adrenal gland.

pH gradient (IPG) strips (pH 3–10 nonlinear) were rehydrated at 50 V for 12 h at 20 °C in 200 μg of whole cell protein extract. Isoelectric focusing (IEF) was carried out sequentially with a Bio-Rad Protean IEF Cell. The equilibrated strips were transferred onto 12% (w/v) acrylamide sodium dodecylsulfate (SDS) gels. To visualize proteins, gels were impregnated with 0.1% silver nitrate solution for 20 min. The density of silver-stained spots from four different experimental sets was determined by both automatic and manual spot detection, and statistically analyzed with PDQuest software (Version 7.1.1, Bio-Rad, Hercules, CA, USA). Silver-stained protein spots were excised from the stained gel and destained solution for 10 min. The peptide samples were spotted onto stainless-steel sample target plates. Peptide mass spectra were obtained using a matrix-assisted laser desorption ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (AB4700, AB SCIEX™, Foster City, CA, USA) in the positive ion reflector mode. Spectra were processed and analyzed by the Global Protein Server Explorer 3.0 software (AB SCIEX™). This uses the internal Mascot (Matrix Science, UK) program for matching mass spectrometry (MS) and MS/MS data against database information. The data obtained were screened against rat databases downloaded from both NCBI (http://www.ncbi.nlm.nih.gov) and the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sprot). Protein identifications were further confirmed by using the MS-Fit (http//prospector.ucsf.edu) and ProFound (http//www.prowl.rockefeller.edu) programs (Fig. 1A)[12]. To measure the expression of myoglobin using the one-dimensional gel electrophoresis (1-DE), gastrocnemius muscle strips were isolated after specific intervals of cast immobilization and snap-frozen in liquid N_2. The samples were then homogenized in a sample buffer. The homogenate was centrifuged and the supernatant was collected. Proteins (50 μg/lane) were separated on 12% (w/v) polyacrylamide SDS gels and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore; Bedford, MA, USA)[13]. Anti-myoglobin antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). β-Actin antibody was purchased from Sigma (St Louis, MO, USA). Antibody-specific bands were quantified using an image analyzer (BioRad). The present investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol for this study was approved by the Committee of Ethics in Research of the University of Yongin, in accordance with the terms of Resolution 5-1-20, December 2006. Data were expressed as the means±SE or means±SEM. The data were statistically evaluated using Student's t test for comparisons between pairs of groups and by ANOVA for multiple comparisons. A p value of < 0.05 was considered to be statistically significant.

RESULTS

As shown in Table 1, with reference to the weight of the control muscles, the muscle weight of the immobilized hind-limbs significantly decreased in a time-dependent manner (n=25, Table 1). The density of myoglobin was significantly increased after seven days of cast immobilization (10657.6±1222.1%) compared with that of the control group (5535.8±83.4%) (n=5, Fig. 1A, 1B-a). Furthermore, the expression of myoglobin detected in the heart and atrophied

Table 1. Changes in muscle weight of rat gastrocnemius muscles during experimental periods

Muscle weight (g)	Experimental period				
	0 Days	3 Days	7 Days	14 Days	21 Days
Time control	0.88±0.02	1.06±0.04	1.28±0.04	1.60±0.03	1.99±0.06
Non-cast immobilization	0.89±0.02	0.93±0.02	1.08±0.01	1.31±0.01	1.52±0.04
Cast immobilization	0.89±0.01	0.84±0.05	0.70±0.02	0.58±0.04	0.49±0.04

Means±SEM *: vs Control, $p<0.05$

skeletal muscle (Fig. 1B-c) was also significantly increased after seven days of cast immobilization (502.5±150.5%) compared with that of the control group (106.5±4.5%) (n=5–7, Fig. 1B-b). Myoglobin was identified using immunoblotting of rat heart and atrophied skeletal muscle strips (n=4, Fig. 1B-c).

DISCUSSION

Decreased muscle activity due to a decrease of muscle loading, a lengthy period of bed rest, malnutrition, or central nervous injury such as stroke and spinal cord injury, triggers signaling pathways to induce skeletal muscle weakness and atrophy[14–18]. Initial muscle atrophy in rehabilitative areas is especially rapid, with up to one-half of the final extent of atrophy occurring within the first week of immobilization, but the severity of muscle atrophy increases for several weeks after the application of a cast[19, 20]. The structural and functional consequences common to all forms of atrophy are decreased muscle mass and cross-sectional area, attenuated contractile force, and increased fatigability[11, 19]. Furthermore, the elevated degradation of proteins in muscle atrophy is coupled with activation of the ubiquitin-dependent protease pathway. Ubiquitinated proteins are degraded by muscle-specific ubiquitin ligases[21]. Previous studies have suggested that the transcriptional regulation of protein ligases E3s, muscle atrophy F-box (MAFbx), also called atrogin-1, and muscle RING finger-1 (MuRF-1), are enriched in skeletal muscles, and are involved in the initiation and development of skeletal muscle atrophy[10, 22]. In addition, mitogen-activated protein kinases (MAPKs), a family of serine/threonine-specific protein kinases that include extracellular signal-regulated kinase (ERK) 1/2, stress-activated protein kinase/*c-Jun* NH_2-terminal kinase (SAPK/JNK), and p38MAPK[23], are also involved in muscle atrophy[11]. Previously, we found that muscle atrophy induced by cast-immobilization, and by starvation of cultivated cells and of muscle tissues, is accompanied by changed phosphorylation of MAPKs[10, 11]. In this study, we demonstrate that cast immobilization decreased the weight of the gastrocnemius muscle in a time-dependent manner. Furthermore, we demonstrated that cast immobilization increases the expression of myoglobin in the gastrocnemius muscle. It has been shown that serum myoglobin with creatine phosphokinase is a major marker that mediates muscle fatigue in a variety of tissues, including skeletal muscle cells[24]. The plasma myoglobin level is also a useful indicator of skeletal muscle toxicity induced by chemicals[25]. Previous papers have reported that myoglobin gene expression shows significant changes, decreasing at 3 days and increasing at 14 days after denervation[26], and that serum myoglobin increases in muscle diseases such as progressive muscular dystrophy and atrophic conditions[27]. It has been reported that patients with chronic progressive myophaties show intense staining of myoglobin and antioxidant enzymes in immuno-histochemical analysis[28]. This stress is further implicated as a cause of immobilization-induced muscle dysfunction, peripheral nerve injury, and myopathy[29, 30]. These results imply that the increment in myoglobin content adapts to stress induced by physical stress such as immobilization. Therefore, in the present study, we demonstrated the intensity of myoglobin using 2-DE with MALDI-TOF/TOF mass spectrometry as an additional test, and expression of myoglobin content using western blotting to identify the proteins involved in muscle atrophy. However, further systematic studies in the area of physical therapy are needed to confirm the mechanism of myoglobin in muscle atrophy. In summary, the intensity of myoglobin and expression of myoglobin were increased in cast-immobilized gastrocnemius muscles. Our results suggest that cast immobilization-induced atrophy may be mediated by myoglobin in the gastrocnemius muscles of rats.

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