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# Expression of ARPP-16/19 in Rat Denervated Skeletal Muscle

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**It is known that denervation of rat skeletal muscle causes atrophy and this is often adopted as a model for human muscle atrophy. To understand the molecular changes that occur, it is important to identify the profiles of differential gene expression. In the present study, we investigated differentially expressed genes in denervated muscle using DNA microarrays with printed genes preferentially expressed in skeletal muscle. We found that several genes are differentially expressed. Of these genes, ARPP-16/19 (cAMP-regulated phosphoprotein 16/19) is selectively enhanced after denervation. The expression of ARPP-16/19 in denervated muscles starts to increase from two days after denervation surgery. On the other hand, the expression of ARPP-16/19 does not change in hind-limb suspended muscles, such as EDL and soleus muscles. These results suggest that the increase in ARPP-16/19 mRNA expression is regulated by unknown factor(s) secreted from nerves, and not by electrical muscle activity.**

**Key words: ARPP-16, ARPP-19, denervation, DNA microarray, hindlimb suspension, muscle atrophy, muscle regeneration.**

Muscle and peripheral nerves have great influences on each other; so, denervation of skeletal muscle causes a wide range of molecular and cellular modulations in skeletal muscle, including atrophy (*1*). The identification and characterization of genes that are differentially expressed in denervated muscle might provide clues to their roles in muscle atrophy and regeneration (*2*). The expression of many genes, including muscle specific genes, increases or decreases in denervated skeletal muscle (*3*, *4*). From a recent study, it is known that denervation causes a dynamic change in cellular chemical modulation, especially phosphorylation signaling (*5-7*).

We investigated differentially expressed genes in denervated muscle by DNA microarray analysis. This array contained 1,536 clones expressed in mammalian skeletal muscles (*8*). The results reveal that many genes are differentially expressed in denervated skeletal muscles, both EDL and soleus. Of these genes, we noted the ARPP-19 (cAMP-regulated phosphoprotein 19 kDa) gene from the viewpoint of signal transduction.

ARPP-19 is a cAMP-regulated phosphoprotein and a substrate for protein kinase A (PKA) *in vitro* and *in vivo* (*9*, *10*). ARPP-19 has another isoform, ARPP-16, that is a cAMP-regulated phoshoprotein with a molecular mass of 16kDa and lacks the 16 amino acids at the N-terminus of ARPP-19 (*9*, *10*). ARPP-16 and ARPP-19 are derived from the same gene, but little is known about the physiological function of ARPP-16 and ARPP-19. ARPP-19 is expressed ubiquitously in most tissues including skeletal muscle. In contrast, ARPP-16 is found mainly in the basal ganglia (*11*). We studied the expression of ARPP-16/19 during the atrophic process in skeletal muscle.

In the present study, differences in ARPP-16/19 mRNA transcripts between denervated and intact muscles were analyzed after denervation surgery. Also, we studied ARPP-16/ARPP-19 mRNA expression in hindlimb suspended muscles, a model that similarly causes atrophy.

## MATERIALS AND METHODS

*2.1. Animals and Surgical Procedures*—Adult male Wistar rats, 9 weeks of age and weighing approximately 250 g, were used in this study. Animals were anesthetized with nembutal (50 mg/kg). Briefly, the sciatic nerve on the right hindlimb was exposed. To maintain the denervated state for at least 2 weeks, a 5 mm segment of the sciatic nerve was surgically removed (*12*). At various time points, rats were deeply anesthetized and killed by decapitation. EDL and soleus muscles from both denervated (right) and innervated (left) legs were immediately removed, frozen in liquid nitrogen, and stored at –80°C.

*2.2. Hindlimb Suspension*—Biopsy specimens of the left EDL and soleus muscles were obtained under general anesthesia before the experiment. The rats were suspended to prevent the right hindlimb from coming in contact with any surface with their tails. Since the forelimb was allowed contact with the floor, the animals were able to move around and obtain food and water ad libitum.

*2.3. RNA Isolation and Northern Blot Analysis*—EDL and soleus muscles were rapidly excised, frozen in liquid nitrogen, and stored at –80°C. Total RNA was prepared with guanidium thiocyanate as described by Chomczynski and Sacchi (*13*). The total RNA in each sample (10-20 μg) was electrophoresed in a 1.0% agarose gel containing

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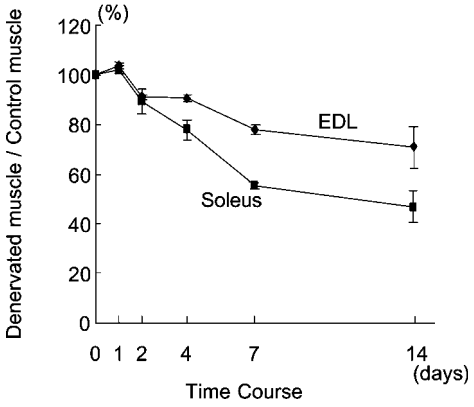


Fig. 1. **Effect of denervation on the wet weight of rat EDL and soleus muscles.** Values are shown as the ratio of the right hindlimb (denervated) muscle weight to the left hindlimb (innervated) muscle weight 1, 2, 4, 7 and 14 days after denervation ($n$ = 3).

formaldehyde and then transferred to a nylon membrane (Biodyne B, KPL). The membranes were hybridized in hybridization solution (ULTRAhyb, Ambion) according to the manufacturer's instructions with $^{32}$P-labeled cDNA fragments encoding human ARPP-19 and human GAPDH. Autoradiographic signals were analyzed and quantified by a Bioimaging Analyzer System (BAS, Fujifilm).

*2.4. Semi-Quantitative RT-PCR*—Total RNA was isolated from frozen muscles as described above. Reverse transcription reactions were performed with Oligo(dT)$_{20}$ primers according to the manufacturer's instructions (Thermoscript™ RT-PCR System, Invitrogen). To standardize the amount of cDNA between normal and denervated muscles, a first PCR was carried out in a total volume of 15 μl containing ExTaq buffer, 0.4 mM dNTPs, 1 unit of ExTaq polymerase (TAKARA), GAPDH gene primers (forward: GCCAAAAGGGTCATCATCTCTG; reverse: CATGCCAGTGAGCTTCCCGT), and gradually increasing amounts of the synthesized cDNAs. The second PCR was carried out in a total volume of 15 μl containing ExTaq buffer, 1 μM gene-specific ARPP-16 primers (Fig. 3a), 0.4 mM dNTPs, 1 unit of ExTaq polymerase (TAKARA) and the same amounts of cDNAs. The PCR products were fractionated in a 4.8% polyacrylamide gel and analyzed using NIH image software.

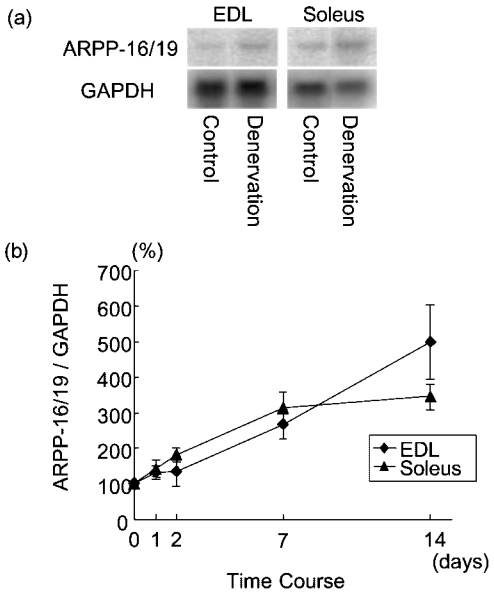


Fig. 2. **Northern blot analyses of ARPP-16/19 mRNA in denervated muscles.** (a) Bands are the signals of ARPP-16/19 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in EDL and soleus muscles two weeks after denervation. (b) The ratios of ARPP-16/19 to GAPDH in EDL and soleus muscles 1, 2, 7 and 14 days after denervation are shown. Values are means ± SE; $n$ = 3.

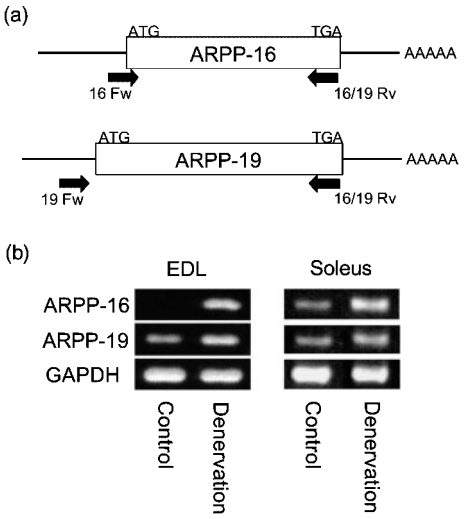


Fig. 3. **Semi-Quantitative RT-PCR of ARPP-16 and ARPP-19 mRNAs in denervated muscles.** (a) ARPP-16 was amplified with 16Fw (GAGTCTAGATATTAAAAGGTG) and 16/19Rv (TCAGCCAGCCAGCTTGCT) primers. The 16Fw primer is in the 5′ UTR of ARPP-16 and does not hybridize with the ARPP-19 cDNA. ARPP-19 was amplified with 19Fw (ATGTCTGCGGAAGTC) and 16/19Rv primers. The 19Fw primer does not hybridize with the ARPP-16 cDNA. (b) The relative expressions of ARPP-16 and ARPP-19 are shown. The sizes of the ARPP-16, ARPP-19 and GAPDH PCR products are 368, 339 and 348 base pairs, respectively. PCRs were performed under the same conditions.

*2.5. Antibody and Immunohistochemistry*—Peptides were synthesized by Sawady Technology. To obtain an ARPP-19–specific antibody, rabbits were immunized with a peptide, MSAEVPEAASAEEQKE corresponding to the N-terminal sequence of rat ARPP-19, conjugated to the KLH protein. Antiserum was purified by affinity chromatography on antigen-coupled Thiopropyl Sepharose 6B (Amersham Biosciences). Serial muscle sections were cut with a cryostat at –25°C, thawed on gelatinized coverslips, and fixed in 4% paraformaldehyde for 15 min. After pre-incubation with phosphate-buffered saline containing 2% bovine serum albumin and 5% heat-inactivated normal goat serum, the sections were incubated with the primary antibody overnight 4°C and the secondary antibody labeled with FITC at room temperature for 40 min. We observed the sections under a Zeiss Axiophot2 microscope (Oberkochen, Germany) with epifluorescence.

*2.6. Statistical Analysis*—All values are expressed as mean ± SE. Statistical analysis was performed by Stu-

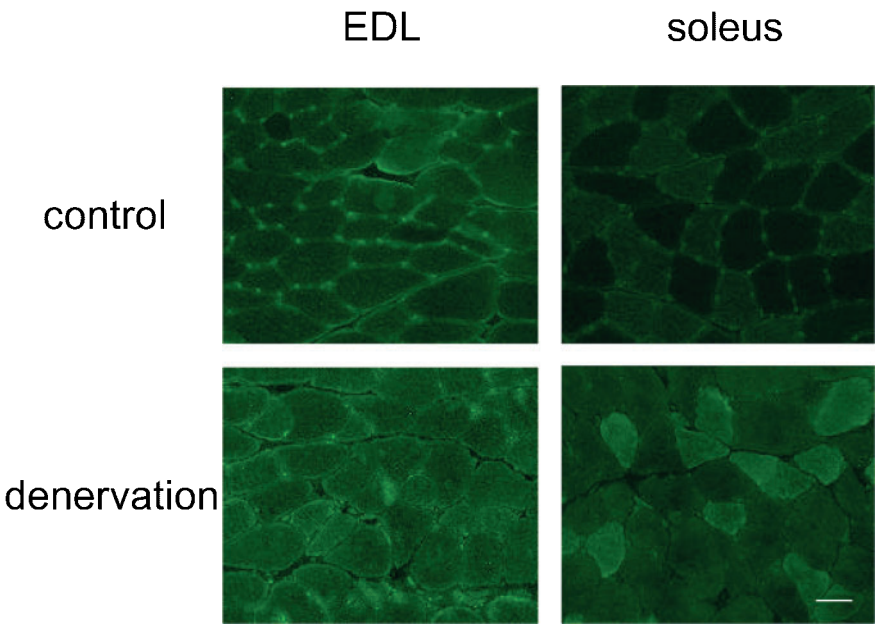


Fig. 4. **Immunohistochemical analysis of the denervated muscles.** Denervated EDL and soleus muscles were removed and frozen seven days after denervation surgery. The anti-ARPP-19 antibodies were detected with second antibodies labeled with FITC. Bar = 50 μm.

dent's t-test. $p < 0.01$ was taken to show a significant difference between values measured in two sample populations.

## RESULTS

The wet weights of the EDL and soleus muscles were unchanged 1 day after denervation and started to decrease constantly after 2 days (Fig. 1). Finally, the weights decreased to 82.4 ± 6.7% for EDL muscles and 63.1 ± 5.7% for soleus muscles one week after denervation, and 70.7 ± 8.4% for EDL muscles and 46.6 ± 6.7% for soleus muscles two weeks after denervation. The decrease in weight was more prominent in the soleus than in the EDL muscles. It is thought that this difference is based on the differences in the composition of fibers in the EDL (fast-twitch fibers) and soleus (slow-twitch fibers) muscles.

We first compared mRNA expression in rat soleus and EDL muscles that had been denervated for seven days with that in control muscles using DNA microarrays. Several genes were found to be differentially expressed. Of these, the changes were most pronounced for the S100A13, cofilin, tropomyosin, protein tyrosine phosphatase receptor type U and ARPP-19 genes. To quantify the changes precisely, we employed Northern blot analysis. For these analyses, the GAPDH gene served as the control gene rather than 18S rRNA because total mRNA in the denervated muscles decreased and the ratio of mRNA to rRNA underwent a large change. One gene was identified as being clearly up-regulated in both denervated EDL and soleus muscles: ARPP-16/19 (Fig. 2a), in addition to nicotinic acetylcholine receptor (28-fold increase).

Next, we analyzed mRNAs prepared from denervated muscles 1 to 14 days after denervation surgery. The expression of ARPP-16/19 in the denervated muscles increased constantly from two days after denervation, reaching 5-fold (EDL) and 3.5-fold (soleus) the starting level after 14 days (Fig. 2b). Because we used a cDNA of the ARPP-19 coding region as a probe, the ARPP-16 and ARPP-19 mRNAs were not separated by Northern hybridization. It is not clear whether ARPP-16 or/and ARPP-19 mRNA increased. Therefore, we employed a

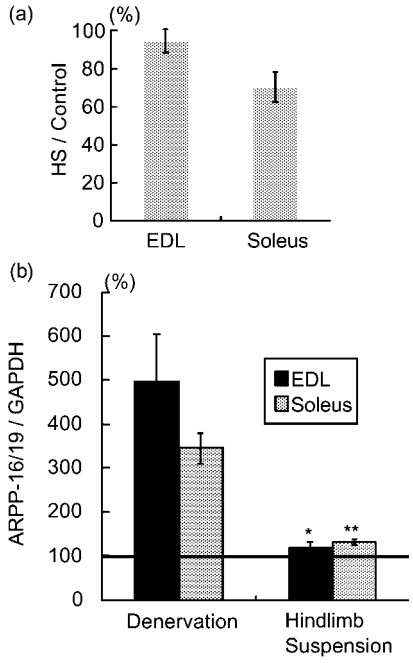


Fig. 5. **Northern blot analysis of ARPP-16/19 mRNA in suspended muscles.** (a) The wet weight ratio of hind-limb suspended (HS) muscles to control muscles was calculated as follows: (suspended right hindlimb muscle / body weight after suspension)/(non-suspended left hindlimb muscle/body weight before suspension). Values are means ± SE; $n = 5$. (b) The ratio of ARPP-16/19 to GAPDH after hindlimb suspension is compared with that after denervation. Values are means ± SE; $n = 3$. *Significantly different from denervated EDL muscles, $p < 0.01$. **Significantly different from denervated soleus muscle, $p < 0.01$.

semi-quantitative RT-PCR method. Fig. 3b shows that the expression of both the ARPP-16 and ARPP-19 mRNAs increased in denervated EDL and soleus muscles. Notably, normal EDL muscles expressed no ARPP-16 mRNA while expression was detected in denervated EDL muscles. Immunohistochemical analysis using the anti-ARPP-19 antibodies shows that the ARPP-19 protein level increased in the cytosol of both denervated EDL and soleus muscles (Fig. 4).

Hindlimb suspension produces atrophy in skeletal muscles (*12*). The wet weights of suspended muscles decreased to 94.6 ± 6.2% (EDL) and 70.2 ± 7.9% (soleus) after two weeks (Fig. 5a). Suspended muscles, especially slow-twitch muscles such as the soleus muscle, are likely to atrophy. We compared the expression of ARPP-16/19 in suspended muscles with that in denervated muscles. The expression of ARPP-16/19 in denervated muscles increased to 497 ± 106% (EDL) and 345 ± 36% (soleus) of the control levels (Figs. 2b and 5b). On the other hand, the expression of ARPP-16/19 in hind-limb suspended muscles did not differ significantly from that in control muscles, *i.e.* 120 ± 13% (EDL) and 132 ± 8% (soleus) (Fig. 5b).

## DISCUSSION

In the present study, we used DNA microarrays to identify genes expressed differentially in denervated skeletal muscles. To investigate gene expression in skeletal muscle, DNA microarrays have many merits because they contain printed genes that are expressed preferentially in mammalian skeletal muscle (*8*). We found that many genes are differentially expressed in denervated muscles, including the ARPP-19 gene.

ARPP-19 is a 19 kDa phosphoprotein regulated by cyclic-AMP. ARPP-19 is expressed ubiquitously and, in the brain, is expressed in embryos much more than at postnatal stages. On the other hand, the expression of ARPP-16 increases with development (*11*). However, the function of ARPP-16/19 is not yet known. In this study, we found that the expression of ARPP-16 and ARPP-19 in skeletal muscles increases after denervation surgery. Denervation causes atrophy and subsequent dedifferentiation in rat skeletal muscle. The levels of myogenic regulatory factors (MRFs) change in denervated muscle. The expression of ARPP-16 is up-regulated during pig skeletal muscle growth (*14*). The increase in ARPP-16/19 mRNA expression may be important in the regeneration and/or atrophy of denervated muscles.

Hindlimb suspension also causes atrophy in skeletal muscle (*15*). In this study, we found that the expression of ARPP-16/19 in hind-limb suspended muscles was the same as that in normal muscles. The functions and characteristics of mature skeletal muscle are maintained by neuronal and hormonal influences, not only by electrical activity (*16*). Neurotrophic factors such as NGF influence the differentiation and proliferation of skeletal muscle (*17*). The above results suggest that the increase in ARPP-16/19 mRNA expression is regulated by unknown factor(s) secreted from nerves, and not by electrical muscle activity. Recently, it was reported that the ARPP-19 protein binds the 3′UTR of the growth-associated protein-43 (GAP-43) mRNA and regulates the stability of GAP-43 mRNA in PC12 cells (*18*). This regulation depends on the NGF stimulation and phosphorylation of ARPP-19.

In conclusion, the increase in ARPP-16 and ARPP-19 expression in denervated skeletal muscles has been demonstrated. The expression and posttranscriptional modification of ARPP-16/ARPP-19 might play an important role in muscle atrophy and/or regeneration.

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