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# Comparative decline of the protein profiles of nebulin in response to denervation in skeletal muscle

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ABSTRACT

The sliding filament model of the sarcomere was developed more than half a century ago. This model, consisting only of thin and thick filaments, has been efficacious in elucidating many, but not all, features of skeletal muscle. Work during the 1980s revealed the existence of two additional filaments: the giant filamentous proteins titin and nebulin. Nebulin, a giant myofibrillar protein, acts as a protein ruler to maintain the lattice arrays of thin filaments and plays a role in signal transduction and contractile regulation. However, the change of nebulin and its effect on thin filaments in denervation-induced atrophic muscle remains unclear. The purpose of this study is to examine the content and pattern of nebulin, myosin heavy chain (MHC), actin, and titin in innervated and denervated tibialis anterior (TA) muscles of rats using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), densitometry and electron microscopic (EM) analyses. The results revealed that denervation induced muscle atrophy is accompanied by decreased nebulin content in a time-dependent manner. For instant, the levels of nebulin in denervated muscles were markedly ($P < 0.05$) decreased, about 24.6% and 40.2% in comparison with innervated muscle after denervation of 28 and 56 days, respectively. The nebulin/MHC, nebulin/actin, and nebulin/titin ratios were decreased, suggesting a concomitant reduction of nebulin in denervated muscle. Moreover, a western blotting assay proved that nebulin declined faster than titin on 28 and 56 days of denervated muscle. In addition, EM study revealed that the disturbed arrangements of myofilaments and a disorganized contractile apparatus were also observed in denervated muscle. Overall, the present study provides evidence that nebulin is more sensitive to the effect of denervation than MHC, actin, and titin. Nebulin decline indeed resulted in disintegrate of thin filaments and shortening of sarcomeres.



## 1. Introduction

Skeletal muscle produces efficient contractile force because they contain thin and thick filaments of well-defined lengths that are organized into regular, symmetric arrays that interdigitate. Filament length is an important aspect of muscle function because a muscle generates force in proportion to thin and thick filament overlap. Nebulin is a giant protein (Mw 700–800 kDa) expressed in skeletal muscle, and covers up 2–3% of the myofibrillar protein mass [1,2]. Although nebulin was discovered over two decades ago, it remains one of the least-understood molecules of striated muscle [2]. In the sarcomere, nebulin spans the entire length of the thin filaments: its N-terminal region extends to the pointed ends of thin filament, and its C-terminal region is anchored at the Z-line lattice [3,4]. Alternative splicing in the central and C-terminal regions

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results in the expression of various nebulin isoforms in different skeletal muscle types [5]. Interestingly, the molecular size of nebulin isoforms correlates with thin filament length variations in different skeletal muscles, and thus these data support the idea that nebulin acts as a protein ruler to specify the lengths of thin filaments [6,7].

The human nebulin gene, on chromosome 2q21.2–2q22, is known to be associated with autosomal recessive nemaline (rod-like) myopathy. The majority of nebulin mutations are frameshifts due to small deletions or insertions; also common are point mutations causing premature stop codons or abnormal splicing [8–10]. This rare musculoskeletal disease is characterized by muscle weakness and the presence of nemaline bodies in the skeletal muscle fibers [10]. However, to devise the therapies against nebulin-nemaline myopathy remains a great challenge. Hindlimb disuse atrophy is induced by a variety of stimuli, such as denervation of the sciatic nerve [11–14], joint immobilization [15], and suspension unloading [16]. Several studies have focused attention on changes in the protein components of skeletal muscle during atrophying process [11,12]. For example, we previously found that increased reduction of titin than that of MHC and actin following denervation affects sarcomeric integrity [11].

To our knowledge, a limited number of studies have examined the changes of nebulin induced by denervation. Here, we used denervation as an experimental model since the atrophy occurs profoundly and quickly [11,13]. Therefore, this study aims to investigate the effect of denervation-induced muscle atrophy on the protein level of nebulin, and also investigate the changes of nebulin in pathological implications in atrophic muscle.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats (8 weeks old) with a mean body weight of 200–250 g were obtained from the Animal Research Laboratory of National Taiwan University (Taipei, Taiwan). The rats were transferred to the Animal Care Facility Centre of Taipei Medical University and housed for 1 week to allow them to acclimatize. Rats were maintained on normal rat chow (PMI Nutrition International, Brentwood, MO, USA) and provided water *ad libitum* throughout the entire experimental period. They were exposed to a reverse light condition of 12: 12 h light: darkness each day with a constant room temperature. Handling of the animals was in accordance with the guidelines established by the Taipei Medical University Ethical Committee for Laboratory Animals.

### 2.2. Denervation procedure

Surgery was performed on adult rats under 35 mg/kg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario, Canada) anesthesia. The unilateral right hindlimb was denervated by excision of 8–10 mm of the sciatic nerve and the contralateral left leg served as a control without nerve sectioning. Atrophy of the right leg muscles was allowed to progress for 0 ($n = 3$), 7 ($n = 6$), 28 ($n = 12$), and 56 ($n = 18$) days following denervation, respectively.

### 2.3. Preparation of muscle sample

After the experimental periods, the TA muscles from both the right (denervated) and left (innervated) legs were immediately snap-frozen in liquid nitrogen, and samples were prepared for SDS-PAGE analysis [11]. Briefly, frozen muscles were pulverized to a fine powder in liquid nitrogen using a mortar and pestle, and the tissue powders were homogenized in a Teflon/glass homogenizer in ice-cold lysis buffer (5 mM EDTA, 30 μg/ml leupeptin, 100 μg/ml PMSF, and 20 mM Tris-base; pH 7.5) on ice. We carefully and thoroughly removed out the collagen fibers around the skeletal muscle and then used the same muscle mass to volume dilution (1 mg muscle tissue/15 μl of lysis buffer). Also, the levels of protein in the myofibrils was measured with a protein assay kit (Bio-Rad, Hercules, California, USA), and the concentration was adjusted to 4 mg/ml with ice-cold lysis buffer. Consequently, myofibrils were solubilized with an equal volume of 2 × SDS sample buffer (2 mM EDTA, 2% SDS, 1.2 M β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, and 20 mM Tris-base; pH 8.0) and heated to 50 °C for 20 min [11].

### 2.4. SDS-PAGE and densitometry

Low percentage SDS–PAGE was employed to investigate high molecular-weight proteins in rat muscles. In order to analyze the actin, MHC, nebulin, and titin (MW from 42 to 3000 kDa), a step gradient minigel with an ambiguous interface was made according to Chen and coworkers [17]. A loading range of each sample was electrophoresed on a “calibration gel”, and then the gel was stained by Coomassie brilliant blue R-250 as described previously [11]. Densitometric analysis was performed with a Photo-Print Digital Imaging System (IP-008-SD; Vilber Lourmat, Marne-la-Vallée, Cedex, France) with analytic software (Bio-1D Light, V 2000). The optical density integrals (ODIs) of nebulin, MHC, actin, and titin were measured for each loading volume, and the slope of the relationship between the ODI and loading volume was determined by linear regression analysis. Also, the ratios of nebulin to MHC (nebulin/MHC), nebulin to actin (nebulin/actin), and nebulin to titin (nebulin/titin) were calculated as the slope of the nebulin ODI/loading volume divided by the slope of MHC ODI/loading volume, the slope of actin ODI/loading volume, and the slope of titin ODI/loading volume, respectively. The HiMark™ unstained high-molecular-weight protein standard (Invitrogen, Carlsbad, California, USA) served as a reference band.

### 2.5. Western blotting analysis

For immunoblot analysis, gels were transferred to a polyvinylidene fluoride membrane as previously described [17]. After blocking, the membrane was reacted with the primary antibodies of mouse anti-titin (clone T11) or anti-nebulin (clone NB2) monoclonal antibody (Sigma, St. Louis, MO, USA). The primary antibody was omitted in the negative control. After washing, the strips were incubated with biotin-conjugated rat serum-adsorbed, and affinity-purified secondary antibody (Vector, Burlingame, California, USA). Following washing, peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark) was added. Subsequently, positive bands were visualized using hydrogen peroxide as the substrate and diaminobenzidene as the chromogen.

### 2.6. Electron microscopy

In this study, we performed electron microscopy to investigate the effect of denervation on the morphological characteristics of myofibrils as previously described our paper [11]. Under anesthesia, the 56-day denervated rats were perfused from the left ventricle of the heart with normal saline and then a mixed aldehyde solution composed of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The TA muscles were removed and postfixed in the same fixative for 4 h at 4 °C. Subsequently, the samples were cut longitudinally with the knife-edge parallel to the muscle fiber and osmicated using 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2). Samples were dehydrated in an ethanol