Influence of electrical stimulation on calpain and ubiquitin-proteasome systems in the denervated and unloaded rat tibialis anterior muscles

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The influence of electrical stimulation on calpain and ubiquitin-proteasome systems was examined in the denervated and unloaded tibialis anterior muscles of male Wistar rats. Animals were divided into 5 groups: control, denervation, denervation plus electrical stimulation, unloading, and hindlimb unloading plus electrical stimulation groups. Due to denervation and unloading for 14 days, muscle atrophy markedly occurred in the denervated and unloading animals, and the atrophy in the former was significantly more severe than that in the latter. In the denervated muscle, the atrophy was significantly attenuated by the electrical stimulation, but not in the unloaded muscle. Overexpression of calpain-2 and ubiquitinated proteins was observed only in denervated muscles. In the unloaded animals, though the expression level of calpain-2 appeared to be slightly higher than that in the control, the expression level of ubiquitinated proteins was almost the same as that in the control. The overexpression of calpain-1, calpain-2, and ubiquitinated proteins in the denervated muscle was inhibited by the electrical stimulation. However, there was no difference in these expressions between the unloaded and unloaded plus electrical stimulation groups. The mechanism of the preventive effect of the electrical stimulation on muscle atrophy might differ between the denervated and unloaded muscles.

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Introduction

Muscle atrophy occurs in denervated, disused and unloaded skeletal muscles. Transection of the sciatic nerve (denervation model) and hindlimb unloading by tail suspension (unloading model) have been widely used as animal experimental models for muscle atrophy in the hindlimbs (Dow et al., 2004; Fujita et al., 2011a,b). It has been reported that the intensity and pattern of the muscle atrophy largely depend on the manner used to induce the muscle atrophy and the muscle fiber types (Russo et al., 2007; Zhang et al., 2010). Skeletal muscle fibers are categorized into slow-twitch oxidative (type I), fast-twitch oxidative-glycolytic (type IIA), and fast-twitch glycolytic (type IIB) types, according to the contractile and metabolic properties (Punkt et al., 2004). In the denervation model, atrophy occurred more severely in the muscle composed mainly of type IIB fibers than that composed mainly of type I and IIA fibers (Russo et al., 2007). In the unloading model, on the other hand, the atrophy of the muscle composed mainly of type IIB fibers was lighter than that composed mainly of type I and IIA fibers (Desplanches et al., 1987; Zhang et al., 2010).

In the normal muscle, protein synthesis and protein degradation are well balanced, but the muscle atrophy would occur if the protein degradation occurred to a greater degree than the protein synthesis (Thomason et al., 1989). It has been reported that protein degradation in skeletal muscles is mediated mainly by the calpain and ubiquitin-proteasome systems (Voisin et al., 1996; Jackman and Kandarian, 2004; Talbert et al., 2013), and it was suggested that calpain and ubiquitin–proteasome systems might be differently activated in the denervated and unloaded muscles.

Electrical stimulation has been widely applied clinically as a countermeasure to attenuate muscle atrophy (Sheffler and Chae, 2007; Adams et al., 2011). Some authors reported that electrical stimulation was effective in ameliorating the muscle atrophy (Roy et al., 2002; Kim et al., 2007; Fujita et al., 2011a,b), while others stated that the electrical stimulation could not prevent the atrophy of the muscles (Dow et al., 2006, 2007; Boonyarom et al., 2009). These findings suggest that the effect of the electrical stimulation on the muscle atrophy might differ according to the measures used to induce the atrophy, and also on the muscle fiber types (Misawa et al., 2001; Boonyarom et al., 2009). Thus, it may be possible...
that the influence of the electrical stimulation on the calpain and ubiquitin-proteasome systems might also differ according to the measures used in the experiments and the muscle fiber types. In the present study, therefore, the influence of electrical stimulation on these two proteolysis systems was examined in the rat tibialis anterior muscle, by using the denervation and unloading animal models.

Materials and methods

Animals

Thirty 12-week-old male Wistar rats, weighing 180–200 g, were used in the present study. These rats were randomly divided into 5 groups: control (Cont, n = 6), denervation (Den, n = 6), denervation plus electrical stimulation (Den + ES, n = 6), hindlimb unloading (HU, n = 6), and hindlimb unloading plus electrical stimulation (HU + ES, n = 6) groups. Each animal in these groups was housed in a separate cage, and maintained in a controlled environment (22 ± 2 °C in a 12:12-h light-dark cycle). The animals were allowed access to food and water freely throughout the experiments. The study was approved by the Institution Animal Care and Use Committee and carried out according to the Animal Experimentation Regulations of our institute.

Sciatic nerve denervation

In the Den and Den + ES groups, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). The bilateral sciatic nerves were surgically exposed, transected with microdissection scissors at mid-thigh level, and approximately 1 cm of the distal stump of the sciatic nerve was removed. Immediately after the operation, the skin wounds were closed with 4–0 suture.

Hindlimb unloading

Hindlimb unloading was carried out for 14 days, according to the Morey technique (Morey et al., 1979). In the HU and HU + ES groups, the tail was wrapped in a tape and suspended by a string. The suspended height was adjusted to allow the forelimbs to make contact with the floor in order to move freely, and to prevent the hindlimbs from weight-bearing on the floor.

Electrical stimulation

In the Den + ES and HU + ES groups, electrical stimulation was carried out by using an electrical stimulator (SEN-3310, Nihon Kohden, Tokyo, Japan). Two surface electrodes coated with electrode paste were placed on the skin covering the mid-belly of the tibialis anterior muscle under anesthesia as described above. Rectangular waves with pulse duration of 1 ms and 100 Hz were used in both groups. The animal was fixed on the apparatus, and the ankle dorsiflexion force elicited by the electrical stimulation was measured to maintain the same contractile tension of the tibialis anterior muscle. The threshold of the nerve fibers becomes higher in the denervated muscle (Ashley et al., 2005; Russo et al., 2007). Therefore, in the Den + ES group, stronger electrical stimulation was required to elicit the same level of contractile tension as in the HU + ES group. It was reported that muscle contractile tension elicited by the electrical stimulation is important to attenuate the muscle atrophy (Russo et al., 2007), and the effect of the electrical stimulation depends on the strength of the muscle contraction (Dennis et al., 2003; Dow et al., 2007). Thus, in the present study, with regard to the electrical stimulation, though the duration and rate by the same pulse were used in the Den + ES and HU + ES groups, the electric current was set to elicit the same level of the dorsiflexion force (1.5 N) in both groups. Twenty times electrical stimulation for 1-s with a 2-s interval were regarded as a single set, and 5 consecutive sets were carried out, followed by 5 min of rest. This stimulation protocol was performed twice a day, 6 days a week, and it was performed for 14 days.

Muscle sample preparation

After 14 days of the experiments, the animals were anesthetized as described above, the tibialis anterior muscles were surgically removed and wet weight was measured. The samples were immediately frozen in acetone cooled by dry ice and stored at −80 °C until analysis.

ATPase and SDH histochemistry

For the histochemical observations, the belly of the muscle was attached to the cryostat chuck, and serial transverse sections (12 μm thick) were cut on a cryostat microtome (CM1510S, Leica Instruments, Heidelberg, Germany) at −20 °C. These sections were mounted on glass slides and dried at room temperature for 30 min. For the adenosine 5′-triposphosphate (ATPase) histochemistry, the sections were preincubated in barbital acetate buffer (pH 4.3) for 5 min at room temperature and rinsed in 0.1 M barbital buffer containing 0.18 M CaCl₂ (pH 9.4) for 30 s. They were incubated in 0.1 M barbital buffer containing 0.18 M CaCl₂ and 4 mM ATP (pH 9.4) for 45 min at room temperature. These sections were then treated with 1% CaCl₂ and 2% CoCl₂ for every 3 min and washed in 0.01 M sodium barbital. After washing in distilled water, the sections were colored by 1% ammonium sulfide. For the succinate dehydrogenase (SDH) histochemistry, these sections were incubated in 0.05% nitroblue tetrazolium and 0.05 M sodium succinate in 0.05 M phosphate buffer (pH 7.5) for 45 min at 37 °C. These sections with ATPase and SDH histochemistry techniques were observed under an ordinary light microscope (BX-51, Olympus, Japan), and the images were obtained with a CCD camera (VB-7010, Keyence, Japan).

Quantitative analysis of the cross-sectional area of each muscle fiber type

The images stained for ATPase activity were used to measure cross-sectional area of each muscle fiber type. Each muscle fiber was matched for ATPase and SDH staining and categorized as type I, IIA, or IIB. The muscle fibers that stained deeply with ATPase histochemistry were treated as type I. The fibers that stained faintly with ATPase and deeply with SDH histochemistry were treated as type IIA. The fibers that stained moderately with ATPase and faintly with SDH histochemistry were treated as type IIB. Photographs (×200) were taken from each section with a digital camera (Olympus, Tokyo, Japan) attached to a light microscope (Olympus, Tokyo, Japan). In order to determine the muscle fiber type composition, a measuring field was set over the entire muscle cross section. At least 100 randomly-selected cross sectional areas of each muscle fiber type were investigated. In these sections, each type of muscle fibers was outlined by using Adobe Photoshop software (Adobe Systems, Mountain View, CA, USA). This operation was performed by one practiced researcher (first author). If muscle fiber which was not round in shape was found, these fibers excluded from the data. The images were analyzed using the ImageJ software program (NIH, Bethesda, MD, USA).

Western blot

The frozen muscle samples were homogenized in ice-cooled homogenizing buffer consisting of 50 mM HEPES (pH 7.4), 0.1%...
Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na4P2O7, 100 mM β-glycerophosphate, 25 mM NaF, 1 mM Na2VO4, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 0.3 μg/ml aprotinin. The homogenates were centrifuged at 10,000 × g for 30 min at 4 °C. The total protein concentration was determined by a protein determination kit (BioRad, Hercules, CA, USA). The homogenates were solubilized in sample loading buffer consisting of 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, and 0.02% bromphenol blue, and boiled for 5 min at 60 °C. Fifty micrograms of sample protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, they were transferred to polyvinylidene fluoride membrane. After an overnight blocking step in 0.3% skimmed milk in PBST, the membranes were incubated with anti-calpain-1 (Cell Signalling, Danvers, MA, USA), anti-calpain-2 (Cell Signalling, Danvers, MA, USA), and anti-ubiquitin (Stressgen, Plymouth Meeting, PA, USA) at 4 °C overnight. The membranes were then incubated for 60 min at room temperature with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (GE Healthcare, Amersham, NJ, USA). The signals were detected by using a chemiluminescent system (ECL plus, GE Healthcare, Amersham, NJ, USA) and analyzed with an image reader (LAS-1000, Fujifilm, Tokyo, Japan).

Statistical analysis

The data were expressed as mean ± SEM. Significant differences between the groups were analyzed using one-way analysis of variance followed by Tukey’s post hoc test. Student’s t-test was used to compare the expression level of ubiquitinated proteins between 7 and 14 days after the experiments. Statistical significance was set as P<0.01.

Results

Muscle wet weight and relative muscle mass

The values of the muscle wet weight in the Den, Den + ES, HU, and HU + ES groups were significantly less than that in the Cont group (Table 1), and the value in the Den group was significantly less than that in the HU group. The value in the Den + ES group was significantly larger than that in the Den group. However, there was no significant difference between the HU and HU + ES groups. The values of the relative muscle mass in the Den and Den + ES groups were significantly less than that in the Cont group, and the value in the Den group was significantly less than that in the HU group. Although the value in the Den + ES group was significantly larger than that in the Den group, there was no significant difference between those in the HU and HU + ES groups.

Muscle fiber cross-sectional area

On the basis of ATPase and SDH histochemical techniques, the tibialis anterior muscles were composed of type I, IIA, and IIB fibers (Fig. 1). Regarding the muscle fiber type distribution, there was no significant difference between all groups.

The cross-sectional areas of all fiber types in the Den, Den + ES, HU, and HU + ES groups were significantly smaller than that in the Cont group (Fig. 2). As to type I and IIA fibers, there was no notable difference between the HU and Den groups. The values of type I and IIA fibers in the HU + ES group were evidently larger than that in the HU group. The value of type IIB fiber in the Den group was remarkably less than that in the HU group. The value of type IIB fiber in the Den + ES group was markedly larger than that in Den group. However, there was no major difference between the HU and HU + ES groups.

Expression level of calpain-1 and calpain-2

It appeared that the expression level of calpain-1 in the Den group was higher than that in the Cont and HU groups (Fig. 3A). Although the expression level in the Den + ES group was significantly lower than that in the Den group, there was no significant difference between the HU and HU + ES groups. The expression level of calpain-2 in the Den group was evidently higher than that in the Cont group (Fig. 3B), but the expression level in the Den + ES group appeared to be slightly lower than that in the Den group. The expression level in the HU group appeared slightly higher than that in the Cont group, and significant difference between the HU and HU + ES groups was not noted.

Expression level of ubiquitinated proteins

The expression level of ubiquitinated proteins in the Den group was clearly higher than that in the Cont and HU groups (Fig. 4A). The expression level in the Den + ES group was significantly lower than that in the Den group. Although evident muscle atrophy occurred in the HU group, no difference was observed in the expression level of ubiquitinated proteins at 14 days between the Cont and HU groups. Therefore, the expression level at 7 days in the HU group was examined. The expression level at 7 days was significantly higher than that at 14 days (Fig. 4B).

Discussion

Muscle atrophy induced by denervation and hindlimb unloading

In the rat tibialis anterior muscle, the loss of wet weight and relative mass was more severe in the denervation model than in the unloading model. According to the ATPase and SDH histochemical techniques, type I, IIA, and IIB fibers were identified in the rat tibialis anterior muscle. Due to denervation and hindlimb unloading, cross-sectional area of these 3 types became evidently smaller than that in the Cont group. Although there were no significant differences in the area of type I or IIA fibers between the denervation and unloading models, the area of type IIB fiber in the denervation model was evidently smaller than that in the unloading model. It was reported that in the denervation model, muscle atrophy occurred more severely in the fast twitch muscle than in the slow twitch muscle (Russo et al., 2007), while in the unloading model, muscle atrophy in the fast-twitch muscle was less than that in the slow twitch muscle (Desplanches et al., 1987; Zhang et al., 2010). In the present study, type IIB fibers in the denervation model severely atrophied, while the atrophy in the unloading model was evidently lighter than that in the denervation model. The tibialis anterior muscle in the unloading model was usually kept in a stretched position, because the ankle plantar flexion was caused by foot drop (Loughna et al., 1986; Riley et al., 1990). It has been reported that the atrophy in the fast twitch muscle depends on the length, and the more the muscle is stretched, the more its atrophy is prevented (Coldspink, 1977; Baker and Matsumoto, 1988; Ohira et al., 1997). Because the tibialis anterior muscle consisted mainly of fast twitch fibers in the present study, the atrophy of the type IIB fibers in the unloading model was attenuated through stretching. On the other hand, when the sciatic nerve was transected, the rats always crouched and their whole plantar including the heel touched to the floor (Nagai et al., 2011). Under this condition, the tibialis anterior muscle is kept in a shortened position with the ankle dorsiflexion. This might be one of the reasons why type IIB fibers in the denervation model atrophied more severely than that in the unloading model. The difference in muscle wet weight and relative muscle
mass among the models might largely depend on the atrophy of the type IIB fibers.

Expression levels of calpains and ubiquitinated proteins

It has been reported that calpain and ubiquitin-proteasome systems are the main pathways to degrade myofibrillar proteins in skeletal muscle (Voisin et al., 1996; Jackman and Kandarian, 2004). Calpain is one of the Ca²⁺-dependent proteases. On the basis of sensitivity for Ca²⁺, it is further divided into μ-calpain (calpain-1) and m-calpain (calpain-2) (Thompson et al., 2000). Calpain-1 is activated with Ca²⁺ level at 3–50 μM, and calpain-2, at 400–800 μM, respectively (Thompson et al., 2000). Wagatsuma (2007) reported that in the denervated muscle, the activity of calpain-1 appeared to increase until 5 days after the denervation, and from 10 to 30 days, it had gradually decreased. At the same period, the activity of calpain-2 appeared to increase. In the unloading muscle, Enns et al. (2007) reported that the activity of calpain-1 began to increase at 12–24 h after the start of unloading, and at 9 days, it began to decrease, but the activity of calpain-2 did not change. It is suggested that since the Ca²⁺ level in muscle fibers increases as the atrophy progress, calpain-1 might have been first activated, and subsequently calpain-2 might be activated (Tompa et al., 1996). On the other hand, it is said that normal myofibrillar proteins are not degraded by the ubiquitin-proteasome system (Solomon et al., 1998), and when ubiquitin binds to myofibrillar proteins, the ubiquitinated proteins are degraded by 26S proteasome (Lecker et al., 1999). In the present study, therefore, the expression level of the

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>Den</th>
<th>Den + ES</th>
<th>HU</th>
<th>HU + ES</th>
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<tr>
<td>Body weight (g)</td>
<td>270 ± 15</td>
<td>256 ± 14</td>
<td>241 ± 11</td>
<td>215 ± 24</td>
<td>208 ± 6</td>
</tr>
<tr>
<td>Muscle wet weight (mg)</td>
<td>480 ± 35</td>
<td>272 ± 34</td>
<td>360 ± 25</td>
<td>372 ± 48</td>
<td>381 ± 22</td>
</tr>
<tr>
<td>Relative muscle mass (mg/g)</td>
<td>1.77 ± 0.09</td>
<td>1.06 ± 0.1</td>
<td>1.5 ± 0.09</td>
<td>1.73 ± 0.14</td>
<td>1.79 ± 0.1</td>
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Values are expressed as the mean ± SEM. Cont, control group; Den, denervation group; Den + ES, denervation plus electrical stimulation group; HU, hindlimb unloading group; HU + ES, hindlimb unloading plus electrical stimulation group.

* Significantly different from the Cont group at P<0.01.
† Significantly different from the Den group at P<0.01.

Fig. 1. Transverse sections of the tibialis anterior muscle assayed for ATPase pretreated at pH 4.4 (A–F) and SDH (B) stains. A, B: Cont group; C: Den group; D: Den + ES group; E: HU group; F: HU + ES group. I: type I fiber; IIA: type IIA fiber; IIB: type IIB fiber. Scale bar = 50 μm.
Fig. 2. Cross-sectional area of type I (A), type IIA (B), and type IIB (C) fibers in the tibialis anterior muscle. Values are presented as the mean ± SEM. *, †, and ‡ are significantly different from the Cont, Den, and HU groups, respectively, at P < 0.01.

Fig. 3. The expression levels of calpain-1 (A) and calpain-2 (B) in the tibialis anterior muscle and representative western blot. These values are calculated as the fold changes relative to the Cont group and presented as the mean ± SEM. a.u. is arbitrary unit. * and † are significantly different from the Cont and Den groups, respectively, at P < 0.01.

Fig. 4. The expression levels of ubiquitinated proteins (A) in the tibialis anterior muscle and representative Western blot. The comparison of expression levels at 7 and 14 days after the unloading (B). The values above are calculated as the fold changes relative to the Cont group and presented as the mean ± SEM. a.u. is arbitrary unit. * and † are significantly different from the Cont and Den groups, respectively, at P < 0.01. ‡ is significantly different from the HU groups on day 7 after unloading, at P < 0.01.
ubiquitinated proteins was measured and used as an indicator of the ubiquitin-proteasome system.

At 14 days after the denervation and the unloading, the expression level of calpain-1 in the denervation model was significantly higher than that in the unloading model. Additionally, the level of calpain-1 in the denervation model appeared to be higher than that in the control. The expression level of calpain-2 in the denervation model was significantly higher than that in the control. Moreover, the level of calpain-2 in the denervation model appeared to be higher than that in the unloading model. The expression level of ubiquitinated proteins in the denervation model was evidently higher than that in the unloading model and the control. It is reported that the ubiquitin-proteasome system is the primary pathway to degrade myofibrillar proteins (Solomon et al., 1998; Lecker et al., 1999). In the present study, the unloading for 14 days induced muscle atrophy, but the expression level of ubiquitinated proteins was almost the same as that of the Cont group. Thus, the expression level of ubiquitinated proteins was measured at 7 days after the start of the unloading. The data showed that the expression level at 7 days was significantly higher than that at 14 days, suggesting that in the unloading model, the expression level began to decrease during the period examined in the present study. These findings taken together suggest that muscle atrophy in the denervation model occurred more severely, probably due to the high level expression of the ubiquitinated proteins for longer time than that in the unloading model. In addition, the expression level of calpain-1 and calpain-2 in the denervation model was higher than that in the unloading model.

**Influence of electrical stimulation on muscle atrophy and proteolysis systems**

In the present study, the preventive effects of the electrical stimulation on muscle atrophy were evident in the denervated tibialis anterior muscle. Wet weight, relative mass, and cross-sectional area of type IIB fibers in the denervated muscle treated with electrical stimulation were significantly larger than those in the denervated muscle without electrical stimulation. Since the tibialis anterior muscle consists mainly of type IIB fibers, the preventive effect of the electrical stimulation on type IIB fibers might result in the effect on the tibialis anterior muscle. In the denervated muscles, contraction does not at all occur, and only by the electrical stimulation, can the muscle contract. As shown in the present study, the expression level of ubiquitinated proteins in the denervated muscle was evidently suppressed by the electrical stimulation. In addition, due to the stimulation, the expression level of calpain-1 and calpain-2 appeared to slightly decrease. These findings suggest that muscle contraction elicited by the electrical stimulation can attenuate muscle atrophy through suppression of the calpain and ubiquitin-proteasome systems. While in the unloaded muscle, the preventive effect of the electrical stimulation on atrophy was hardly discerned. The expression levels of calpains and ubiquitinated proteins in the unloaded muscle treated with the electrical stimulation were almost the same as the unloaded muscle without electrical stimulation. Although the expression level of calpain-2 in the unloaded muscle appeared to be slightly higher than that in the Cont group, there was no difference in calpain-1 and ubiquitinated proteins among the unloaded, unloaded with electrical stimulation, and control muscles. Since muscle wet weight, relative muscle mass, and cross-sectional area of type IIB fibers in the unloading model were significantly larger than those in the denervation model, the atrophy might have been considerably reduced through continuous stretching. Thus, the effect of the electrical stimulation in the unloaded muscle might be limited. Only the cross-sectional areas of type I and IIA in the unloaded muscle with the electrical stimulation were larger than those in the unloaded muscle without it. Since the tibialis anterior muscle contained only a few type I and IIA fibers, the effect of the electrical stimulation on atrophy in slow twitch muscle should be examined in other muscles.

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**References**


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