Acupuncture ameliorated skeletal muscle atrophy induced by hindlimb suspension in mice

Akiko Onda a, Qibin Jiao b, Yasuharu Nagano c, Takayuki Akimoto d, Toshikazu Miyamoto e, Susumu Minamisawa b,*, Toru Fukubayashi a

a Graduate School of Sport Sciences, Waseda University, Tokyo, Japan
b Graduate School of Advanced Science and Engineering, Waseda University, Tokyo, Japan
c Department of Health and Sports, Niigata University of Health and Welfare
d Center for Disease Biology and Integrative Medicine, The University of Tokyo, Japan
e Graduate School of Comprehensive Human Sciences, University of Tohoku, Japan

A R T I C L E   I N F O

Article history:
Received 24 May 2011
Available online xxxx

Abstract

Preventing skeletal muscle atrophy is critical for maintaining quality of life, but it is often a challenging goal for the elderly and patients with severe conditions. We hypothesized that acupuncture in place of exercise training is an alternative non-pharmacological intervention that can help to prevent muscle atrophy. To elucidate the effects of acupuncture on skeletal muscle atrophy caused by hindlimb suspension (HS), we performed acupuncture on mice according to two different methods: acupuncture with electrical stimulation (EA: electroacupuncture) and without electrical stimulation (MA: manual acupuncture). A needle was retained in the gastrocnemius muscle for 30 min every day for 2 weeks in the EA and MA groups. In the EA group, 30 min of repetitive electrical stimulation (1 Hz, 1 ms pulse width, 6.5 mA intensity) was also applied. HS significantly reduced muscle mass and the cross-sectional area of the soleus muscles. This HS-induced reduction was significantly improved in the EA group, although the level of improvement remained insufficient when compared with the control group. We found that the mRNA expression levels of atrogin-1 and MuRF1, which play a principal role in muscle-specific degradation as E3 ubiquitin ligases, were significantly increased in the HS group compared to the control group. EA and MA reduced the HS-induced upregulation of atrogin-1 (p < 0.01 in EA and MA) and MuRF1 (p < 0.01 in EA) mRNAs. We also found that the expression levels of PI3K, Akt1, TRPV4, adenosine A1 receptor, myostatin, and SIRT1 mRNAs tended to be increased by HS. EA and MA further increased the HS-induced upregulation of Akt1 (p < 0.05 in MA) and TRPV4 (p < 0.05 in MA) mRNAs. We concluded that acupuncture partially prevented skeletal muscle atrophy. This effect might be due to an increase in protein synthesis and a decrease in protein degradation.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Loss of skeletal muscle mass has a profound effect on a patient’s daily life, especially physical activity. The resulting reduction in physical activity induces further skeletal muscle atrophy, leading to a vicious circle of the atrophic process [1]. Since skeletal muscle has high plasticity, interventions such as exercise training, nutrition, and mechanical stimulation are recommended to prevent skeletal muscle atrophy [2]; it is often hard, however, for the elderly and patients with serious diseases to continue exercise training and even to maintain daily physical activity. As an alternative to exercise training, therefore, a non-pharmacological intervention is urgently required, especially given the rapid aging of our society.

Acupuncture is a branch of traditional East Asian medicine that is widely applied to various diseases [3]. The consensus of the World Health Organization is that acupuncture can be used for stroke rehabilitation, headache, menstrual cramps, osteoarthritis, low back pain, carpal tunnel syndrome, asthma, and other conditions [4,5]. In addition to these indications, electroacupuncture (EA) is used for recovery from skeletal muscle fatigue and musculoskeletal disorders [6–8]. The mechanism of acupuncture has been extensively investigated [9]. EA increases blood flow in and oxygenation of skeletal muscles [10,11] and evokes somatosensory responses of the brain, spinal cord and muscles in humans [12], and these factors are thought to contribute to its ameliorating effect on muscle fatigue. The effect of acupuncture on muscle atrophy, however, has not yet been sufficiently elucidated.
Muscle mass and structure are determined by the balance between protein degradation and synthesis [1]. In the protein degradation pathway, ATP–ubiquitin-dependent proteolysis is the process most responsible for muscle wasting. There are three enzymes involved in the polyubiquitination cascades in this process: E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligase). Recent studies have established that muscle-specific E3 ubiquitin ligases such as atrogin-1 and MuRF1 play critical roles in muscle atrophy [13]. Atrogin-1 contains an SCF complex (Skp, Cull and Roc1) [14] and directly interacts with calcineurin A and α-actinin-2 at the Z-disc [15]. MuRF1 is a member of the Ring finger–B-box-coiled-coil family [16] and interacts with titin in the M-band [17]. Previous studies have demonstrated that the expression levels of atrogin-1 and MuRF1 are increased in atrophic skeletal muscles and that mice deficient in either atrogin-1 or MuRF1 are resistant to muscle atrophy [14,18,19].

The insulin-like growth factor 1 (IGF-1) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is known to play a pivotal role in activating protein synthesis [13,20]. PI3K, which is activated by insulin or IGF, in turn activates Akt, a serin/threonine kinase, and its downstream phosphorylates glycogen synthase kinase 3beta (GSK-3β) and mammalian target of rapamycin (mTOR), thereby inducing hypertrophy [21].

Given all this, we hypothesized that acupuncture is a promising feasible non-pharmacological strategy for preventing skeletal muscle atrophy. In this regard, Takaoka et al. have also demonstrated that EA treatment induced a satellite cell–related proliferative reaction in non-atrophic skeletal muscle through the suppression of myostatin expression [22], myostatin being a potent negative regulator of muscle growth. Therefore, to test our hypothesis, we investigated the effect of acupuncture on skeletal muscle atrophy induced by hindlimb suspension (HS) in mice and the expression levels of muscular-specific mRNAs involved in muscular protein degradation and synthesis.

2. Materials and methods

2.1. Animals and acupuncture conditions

Among the various animal models of skeletal muscle atrophy, including unloading [23,24], immobilization [25], starvation [26], denervation [27], and administration of dexamethasone [28], we selected unloading by HS, because HS is one of the most well-known atrophic models induced by non-weight bearing conditions [29]. HS consisted of tail suspension from the cage ceiling. Mice were housed in independent plastic cages at 20–23 °C with a 12:12-h light–dark cycle with free access to food and water ad libitum.

Twenty-eight male mice (C57 BL/6, 8 weeks; Sankyo Lab Service, Tokyo, Japan) were randomly assigned into four groups; (A) control, (B) untreated hindlimb suspension (HS), (C) HS + manual acupuncture (MA), (D) HS + MA + electrical stimulation (EA) (n = 7/group). Mice in the MA and EA groups were anesthetized with Somnopentyl (10 mg/kg, Kyoritsu Seiyaku, Tokyo, Japan) by intraperitoneal injection so that they would remain in a recumbent position. Both soleus (slow-twitch) and gastrocnemius (fast-twitch) muscles were dissected from the hindlimb. Body and muscle weights were measured shortly after death. Tissues were frozen with liquid nitrogen and stored at −80 °C.

2.2. Sample collection

After the 2-week period of acupuncture treatment, all mice were sacrificed. Both soleus (slow-twitch) and gastrocnemius (fast-twitch) muscles were dissected from the hindlimb. Body and muscle weights were measured shortly after death. Tissues were frozen with liquid nitrogen and stored at −80 °C.

2.3. Cross-sectional area

All samples were cut into 7-μm-thick sections using the LEICA (Wetzlar, Germany) CM1850 cryostat. Sliced samples were immunostained with primary anti-dystrophin antibody (Sigma–Aldrich, St. Louis, MO, USA), with secondary FITC-conjugated antibody mouse IgG (Invitrogen, Carlsbad, CA, USA). Images showing cross-sectional areas (CSA) were taken with a Keyence (Osaka, Japan) BZ9000 microscope, and CSA of cell membranes were measured with Image J software (National Institutes of Health, Bethesda, MD, USA).

2.4. Quantitative reverse transcription-PCR

RNA was extracted using Trizol reagent (Invitrogen), according to the method described in our previous studies [30]. The RNA concentrations and quality were determined by spectrophotometry. To remove contaminating DNA, samples were treated with recombinant Dnase I (DNA-free; Ambion, Austin, TX, USA). RNA was reverse transcribed using the reagent High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The internal control was 18s ribosomal RNA. The sequences of the PCR oligonucleotide primers are listed in Table 1.

2.5. Statistical analysis

The statistical significance of the differences between means was assessed using one-way ANOVA followed by the Tukey–Kramer post hoc test. A probability of less than 5% was considered significant (p < 0.05). Data are expressed as the mean ± SEM. Statistical significance is indicated as “p < 0.05,” “p < 0.01,” “p < 0.001” when compared to the control group, and as “p < 0.05,” “p < 0.01,” “p < 0.001” when compared to the HS group.

3. Results

After 2 weeks of HS, muscle mass and cross-sectional area (CSA) were significantly reduced in the soleus muscle (56% and 45% reductions, respectively) (Fig. 1A and B), and were reduced, though not significantly, in the gastrocnemius muscle (20% and 21% reductions, data not shown), indicating that HS induced muscle atrophy to a greater degree in the soleus than in the gastrocnemius muscle. The present data is consistent with a previous study demonstrating that HS has a greater effect on muscle atrophy in slow skeletal muscles [31]. Therefore, we investigated the effect of acupuncture on HS-induced muscle atrophy in slow skeletal muscles. HS-induced reduction in soleus muscle mass was significantly improved in the MA and EA groups (7% and 10% increases, respectively; Fig. 1A), although this level of improvement was insufficient, as muscle mass was still lower in the MA and EA groups than in the control group (Fig. 1A). Furthermore, HS-induced reduction in the CSA of the soleus muscle was significantly improved in the EA group by 9% (p < 0.05) compared to the HS group (Fig. 1B).
improved in the EA group (18% increase; Fig. 1B), although this level of improvement was insufficient, as CSA was still lower in the MA and EA groups than in the control group (Fig. 1B and C).

Consistent with previous studies [32–34], we found that unloading by HS significantly increased the expression levels of atrogin-1, MuRF1, PI3K, myostatin and SIRT1 mRNAs. At the end of the two-week period of acupuncture treatment, significant increases in mRNA expression levels in the soleus were observed for both atrogin-1 (302% increase compared to control group; Fig. 2A) and MuRF1 (247% increase compared to control group; Fig. 2B). The HS-induced increase in atrogin-1 expression in the soleus muscle was significantly decreased in the MA and EA groups (Fig. 2A), while that in MuRF1 expression was significantly decreased in the EA group alone (Fig. 2B), although these levels of improvement were insufficient when compared with the control group (Fig. 1A and B).

Turning to the protein synthesis pathway of the soleus, the expression level of PI3K mRNA was significantly increased in the HS group (295% increase; Fig. 2C), but was not reduced by MA or EA. On the other hand, although Akt1 mRNA expression was not significantly increased by HS, the expression levels of the Akt1 mRNA group in the soleus muscle were significantly upregulated by MA compared to the control group (213% increase; Fig. 2D).

We also found that the expression of adenosine A1 receptor mRNA was higher in the MA group than in the control and HS groups (Fig. 2E). Furthermore, we found that the expression levels of myostatin and SIRT1 mRNAs were significantly increased by HS (269% and 488% increase, respectively; Fig. 2G and H) but were not affected by acupuncture.

Table 1
Oligonucleotides for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>5′–3′ Sequence</th>
<th>Size (bp)</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrogin-1</td>
<td>Forward CAGCTCGTGAGCGACCTC 244 67731</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse GGCAGTCGAGAAGTCCACTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MuRF1</td>
<td>Forward GACAGTCGACCTTCAACAGCA 194 433766</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse GCCCTACGAGAAGTCCACTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K p85α</td>
<td>Forward GCCAGTGCTATTTGTCTTGG 236 18708</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse ACAACCCGCAGGAGACTCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akt1</td>
<td>Forward ACTGACGACTGTACTGGAAG 116 18708</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse TGTGAGCCATAAAGTGCCACAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine A1R</td>
<td>Forward TATGACGACTGACTGACTGTA 155 11539</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse AGACCTACCTGAGACACCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPV4</td>
<td>Forward CACACCTCGGGAACCTGA 185 93759</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse CACACCTCGGGAACCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myostatin</td>
<td>Forward CACACCTCGGGAACCTGA 185 17700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse CACACCTCGGGAACCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIRT1</td>
<td>Forward ATGACGACTGTACTGGAAG 116 18708</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse TGTGAGCCATAAAGTGCCACAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18s Ribosomal RNA</td>
<td>Forward GACCTGAGAAACGGCTACC 252 19791</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse TGTGAGCCATAAAGTGCCACAT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Muscle weight/body weight and cross-sectional area. After the two-week period of acupuncture treatment, soleus muscle weights were significantly greater in the MA and EA groups than in the HS group (p < 0.05, p < 0.001). CSA was significantly greater in the EA group than in the HS group (p < 0.05). Data is shown as the mean ± SEM. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001 when compared to the control group, and as †p < 0.05, ††p < 0.01, †††p < 0.001 when compared to the HS group. n = 7/group. Scale bar shows 300 µm.
4. Discussion

To the best of our knowledge, our data indicated for the first time that acupuncture partially prevents skeletal muscle atrophy caused by HS. We presume that acupuncture prevents protein degradation and supports muscle synthesis in atrophic skeletal muscles, given that, in the present study, acupuncture downregulated the genes involved in muscle-specific degradation, such as atrogin-1 and MuRF-1, and upregulated the genes involved in protein synthesis, such as Akt1 and TRPV4. Previous studies have demonstrated that acupuncture increases the blood flow in skeletal muscle [10,11] and evokes somatosensory responses of the brain, spinal cord and muscle [12], resulting in a reduction of muscle fatigue. Although we did not examine neurohormonal responses to muscle atrophy.

Fig. 2. mRNA expression levels as measured through quantitative RT-PCR. After the two-week period of acupuncture treatment, in the protein degradation pathway, (A) the proportion of atrogin-1 mRNA was significantly lower in the MA and EA groups than in the HS group (p < 0.001, respectively). (B) The mRNA expression level of MuRF1 was significantly lower in the EA group than in the HS group (p < 0.001, respectively). In the protein synthesis pathway of the soleus, the proportion of (D) Akt1 mRNA was significantly higher in the MA groups than in the HS group (p < 0.05). (F) The mRNA expression level of TRPV4 was significantly higher in the MA group than in the HS group (p < 0.05). mRNA expression levels were analyzed by quantitative real-time PCR. The internal control was 18s Ribosomal RNA. Data is shown as the mean ± SEM. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001 when compared to the control group, †p < 0.05, ‡p < 0.01, §p < 0.001 when compared to the HS group (n = 7).
in the present study, we assume that these factors play a role in preventing muscle atrophy. Importantly, the present study demonstrated that repetitive acupuncture significantly suppressed protein degradation in atrophic muscles at the transcriptional level. Similarly, chronic resistance training [35,36] and amino acid-containing diets [37,38] are known to decrease muscle-proteolytic genes including atrogin-1 and MuRF-1. Therefore, the present data indicate that acupuncture may be an alternative to exercise and/or a supplementary means of preventing muscle atrophy.

Regarding the effect of acupuncture on gene expression in skeletal muscles, Takaoka et al. found by means of an mRNA fingerprinting method and differential display analysis that EA suppressed the expression of the myostatin mRNA [22], a member of the transforming growth factor-β (TGF-β) superfamily. This finding supports our hypothesis that EA has an ameliorating effect on muscle atrophy, although we did not find that acupuncture significantly decreased the expression of the myostatin gene in the atrophic soleus muscle. We did not completely understand why our data was different from that of Takaoka et al. [22]. One possible reason is that we examined the effect of EA on atrophic muscles, whereas Takaoka et al. used healthy muscles. Another is that we examined the soleus muscle, which contains a high proportion of slow-twitch/oxidative fibers which are known to have lower myostatin expression levels than fast-twitch/glycolytic fibers, such as those in the gastrocnemius muscle [32], have; lower myostatin expression levels could make it difficult to identify small changes in myostatin gene expression. In addition, it should be noted that Takaoka et al. demonstrated that acupuncture without electrical stimulation did not significantly suppress the expression of the myostatin gene. Therefore, it is possible that the level of electrical stimulation applied in the present study was not strong enough to suppress the expression of myostatin, as that applied by Takaoka et al. did. It would be intriguing to investigate in a future study whether acupuncture induces a satellite cell-related proliferative reaction and repair in atrophic skeletal muscle.

The present study revealed that EA was more effective at preventing muscle atrophy and reducing the HS-mediated induction of MuRF1 gene expression than MA was. As mentioned above, EA, but not MA, decreased the expression of the myostatin gene [22]. A large quantity of evidence has indicated that electrical stimulation prevents muscle atrophy [39,40]. Recent studies have also demonstrated that electrical stimulation reduces the atrogin-1 expression level in a denervated rat model compared to a control group [41–43]. Nevertheless, it should be emphasized that MA (without electrical stimulation) also improved muscle mass and suppressed HS-mediated induction of the atrogin-1 gene, though MA alone was not sufficient to significantly prevent HS-induced reduction of the cross-sectional area of the soleus muscle. In addition, MA had a stronger effect on the expression levels of Akt1 and TRPV4 mRNAs than EA did. Along the same lines, a recent study has demonstrated that MA, but not EA, increased carnitine in muscle tissue, whereas both EA and MA increased glutathione [44]. MA is known to cause tissue to wind around the needle, leading to activation of the sensory mechanoreceptors [45], but MA without electrical stimulation might be more feasible than EA for many candidates, as it does not require an expensive electrical stimulator, and can therefore be performed at the patient’s bedside.

The molecular mechanisms by which the retention of a needle leads to amelioration of skeletal muscle atrophy will be uncovered in future studies. Although these mechanisms are not yet fully understood, we examined the expression levels of genes that are known to be involved in skeletal muscle regeneration and/or differentiation or to be affected by acupuncture. Among the genes examined in this study, we consider adenosine A1 receptor and TRPV4 to be possible candidates for further investigation on the grounds that they responded significantly to EA or MA. Interestingly, a recent study has established that adenosine A1 receptor is responsible for the anti-nociceptive effects of acupuncture [46]. Another intriguing molecule to be investigated is TRPV4, a member of the TRP channel superfamily [47,48]. TRPV4 is known to be a Ca2+-permeable nonselective cation channel, and it appears to play a mechanosensory or osmosensory role in several musculoskeletal tissues [48]. Since adenosine A1 receptor and TRPV4 are involved in diverse physiological functions in skeletal muscle, it will be intriguing to investigate their roles in the anti-atrophic effect of acupuncture.

The present study indicated that acupuncture is a viable alternative non-pharmacological intervention to prevent and reverse skeletal atrophy. Loss of skeletal muscle mass has a profound effect on the physical-activity aspects of a patient’s daily life, especially among the elderly and patients with serious diseases, for whom exercise training, the recommended means of preventing skeletal muscle atrophy [2], is often difficult. Especially in such cases, acupuncture can serve as an alternative non-pharmacological intervention that is feasible, versatile, and associated with little risk. Further investigations concerning possible molecular mechanisms will provide a better understanding of how acupuncture treatment prevents skeletal muscle atrophy.

Acknowledgments

We thank Professor Takashi Ushida, Mr. Shogo Wada and Ms. Sachiko Ilemune for their help with our technical analysis. This study was supported by grants from the IBUKA Foundation (I.F.) and the Global COE Program of Waseda University (T.F.), the Ministry of Education, Culture, Sports, Science and Technology of Japan (S.M.) and High-Tech Research Center Project for Private Universities (S.M.).

References

Acupuncture ameliorated skeletal muscle atrophy induced by hindlimb suspension in mice.