



Ectopic TWEAKR expression in type I fiber of stroke-prone spontaneously hypertensive rats is related to slow muscle-specific hypotrophy

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ABSTRACT

Aims: Stroke-prone spontaneously hypertensive rats (SHRSP) show significantly lower body weight than normotensive Wistar-Kyoto rats (WKY). Our hypotheses are as follows: weight loss of the skeletal muscle is related to hypertension-related diseases, and muscle hypotrophy is useful as a therapeutic target for hypertension and hypertension-related diseases. In this study, we aimed to investigate the pathophysiological characteristics of muscle hypotrophy in SHRSP to determine the therapeutic target molecule(s).

Main methods: The difference in skeletal muscles in the lower leg between WKY and SHRSP was evaluated mainly through weight/tibial length, histological, gene expression, and protein expression analyses.

Key findings: SHRSP had a significantly lower weight/tibial length in soleus and gastrocnemius, but not in plantaris and tibialis anterior, indicating that muscles consisting of a relatively high amount of slow muscle fiber were affected. This result was confirmed by the histological analysis of soleus, showing that type I fiber mainly decreased the fiber size. Microarray and protein expression analyses showed that the muscle-specific ubiquitin ligase, muscle RING finger 1 (MuRF1), but not atrogen-1, was highly expressed in soleus, but not in plantaris, in SHRSP. TNF-like weak inducer of apoptosis receptor (TWEAKR) was predicted as a MuRF1 up-regulator by Ingenuity Pathway Analysis and immunostained only in type II fiber in WKY but in both type I and II fibers in SHRSP.

Significance: TWEAKR is a type II-specific receptor in the skeletal muscle. Ectopic TWEAKR expression in type I fiber of SHRSP is most likely involved in slow muscle-specific hypotrophy through MuRF1 overexpression.

1. Introduction

The skeletal muscle is an important organ as locomotorium and occupies approximately 40% of the body. Additionally, recent studies have shown the importance of skeletal muscle as the largest endocrine organ secreting myokines, which are muscle-derived proteins [1,2]. Myokines play important roles for organ crosstalk and body homeostasis. Exercise is known as a promoter for myokine secretion, such as interleukin-6 (IL-6) [1] and secreted protein acidic and rich in cysteine (SPARC) [3]. IL-6 and SPARC relieve disease symptoms, such as improvement of insulin secretion [4] and sensitivity [2], and suppression of colon tumorigenesis [3], respectively. Body muscle mass is also related to several diseases, such as heart disease and longevity [5]. Atrophy by cachexia, however, lowers the patients' quality of life. Zhou

et al. showed that suppression of muscle atrophy by cachexia extends the life, although cancer progression cannot be slowed down [6]. Therefore, muscle atrophy brings not only locomotive problem, but also disease problem. It is considered to be a disturbance of myokine secretion. Atrophy is caused by the imbalance between muscular protein synthesis and degradation. Muscular protein degradation is mainly mediated by calpain [7], lysosome (cathepsins) [8], and ubiquitin-proteasome pathways [9]. Under microgravity, the ubiquitin-proteasome pathway is mainly involved in muscular protein degradation [10]. The ubiquitin system is also involved in other atrophic conditions, such as disuse and denervation [11,12]. However, calpains and cathepsins are also observed in atrophy [7,13]. Additionally, calpains and cathepsins are involved in inflammatory myopathies [14]. In contrast, little is known about the mechanism of skeletal muscle hypotrophy and the

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relationship between skeletal muscle hypotrophy and myokines.

Stroke-prone spontaneously hypertensive rats (SHRSP) developed from normotensive Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) in order show severe hypertension and stroke with age. The body weight difference is observed among these hypertensive rats through life. Additionally, blood pressure and body weight show an inverse correlation between WKY, SHR, and SHRSP [15]. Therefore, there is the possibility that the weight difference, especially skeletal muscle mass difference, between SHRSP and WKY may be related to the worsening of hypertension and hypertension-related diseases, such as stroke and kidney disease in SHRSP by possible myokine disturbance. Improvement of skeletal muscle hypotrophic condition in SHRSP may relieve the symptoms of hypertension and hypertension-related diseases.

In the present study, we aimed to investigate the pathophysiological characteristics of muscle hypotrophy in SHRSP to determine the therapeutic target(s). Firstly, skeletal muscle hypotrophy was evaluated by focusing on the difference in the skeletal muscles in the lower leg between WKY and SHRSP through weight and histological analyses. Further investigations were carried out through a comprehensive gene expression analysis with microarray, protein expression, and immunohistochemistry.

2. Materials and methods

2.1. Ethical approval

All experimental protocols conformed to the guidelines published in the "Guide for the Care and Use of Laboratory Animals" of the US National Institutes of Health (8th edition, revised 2011) and were approved by the Institutional Animal Experimentation Committee of Kindai University Faculty of Medicine (approval number: KAME15-26).

2.2. Animals

Male WKY and SHRSP maintained in the Kindai University Life Science Research Institution were used at 12 weeks of age. All animals were housed in the animal center under constant temperature and humidity and were fed a Funabashi SP diet (Funabashi Farm, Chiba, Japan) and tap water *ad libitum*. Rats were anesthetized by inhalation with 4% isoflurane for induction and 1.5%–3% isoflurane for maintenance and then euthanized by exsanguination.

Blood pressure was measured using the tail cuff method. Rats were kept at 38 °C for 10 min and then held without anesthesia with a holding tool during measurement. For histological analysis, skeletal muscle tissues were frozen directly in isopentane-dry ice and then stored at –80 °C until use [16]. For microarray analysis, soleus muscle tissues were placed in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C overnight and then re-placed to new tubes and stored at –80 °C until use. For protein analysis, tissues were frozen in liquid nitrogen and then stored at –80 °C until use.

2.3. Antibodies

Anti-muscle RING finger 1 (MuRF1) and anti-atrogin-1 polyclonal antibodies were purchased from ECM Biosciences (Versailles, KY, USA). Anti-ubiquitin, anti-tumor necrosis factor-like weak inducer of apoptosis (TWEAK) receptor (TWEAKR), and anti-fast myosin skeletal muscle heavy chain antibodies were from Abcam (Cambridge, UK). Anti-CD45 was purchased from Proteintech (Resemont, IL, USA). Anti-GAPDH antibody conjugated with horseradish peroxidase was from Medical & Biological Laboratories (Nagoya, Japan). Secondary antibody against rabbit conjugated with horseradish peroxidase was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). Secondary antibodies against rabbit conjugated with Alexa 594 and mouse conjugated with Alexa 488 were from Jackson ImmunoResearch

Inc. (West Grove, PA, USA). Histofine Simple Stain Rat MAX-PO(R), the secondary antibody, for anti-CD45 antibody was purchased from Nichirei Bioscience (Tokyo, Japan).

2.4. Histopathological analyses

Frozen soleus and plantaris muscles were cut in cryostat (Leica CM3050 S, Leica Microsystems GmbH, Wetzlar, Germany) at –20 °C with 10- μ m thickness of cross section. Hematoxylin-eosin (H&E) staining was carried out after fixing with 4% paraformaldehyde for 10 min. Necrosis and regeneration of muscle fibers were counted in the entire specimen with H&E staining by the following criteria. Necrosis of fibers includes pyknosis, karyorrhexis, and karyolysis of the nuclei and cytoplasmic hypereosinophilia. Regeneration of fibers includes central or enlarged nuclei in the fiber. For inflammation, immunostaining was carried out with anti-CD45 antibody, Histofine Simple Stain Rat MAX-PO(R), and then ImmPACT™ AEC Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA) for staining all leukocytes. The CD45-positive cells were counted in the entire specimen. For type distribution of soleus (types I, IIa, and IIc), ATPase staining after treatment at pH 10.5 solution was carried out [16]. According to ATPase staining, type distribution and cross-sectional area by type were determined using the NIS-Elements D software (Nikon Instech, Tokyo, Japan). The cross-sectional area was measured with more than 150 muscle fibers/rat (n = 6). Immunohistochemistry was done after fixing with 4% paraformaldehyde for 10 min. Specimens were incubated with primary antibodies at 4 °C overnight, and then with secondary antibodies. Images were obtained with Nikon ECLIPSE Ti (Tokyo, Japan).

2.5. RNA isolation, quality check, and microarray and data analyses

Soleus muscle obtained from WKY and SHRSP was homogenized in QIAzol Lysis Reagent (QIAGEN, Chatsworth, CA, USA), and then RNA was isolated with RNeasy Plus Universal kit (QIAGEN). RNA samples isolated were applied on RNA 6000 Nano assay (Agilent, Santa Clara, CA, USA) for RNA quality check. The RNA samples showing > 7.5 of the RNA integrity number were proceeded for microarray analysis. For microarray, all reagents, instruments, and software used were from Thermo Fisher Scientific unless stated otherwise. Samples for microarray were prepared using 100 ng RNA with GeneChip® WT PLUS Reagent Kit according to the manufacturer's procedures. Samples prepared were applied on Affymetrix Clariom S rat, hybridized (45 °C, 60 rpm, 16 h) with GeneChip® Hybridization Oven 645, and then washed with GeneChip® Fluidic Station 450. Hybridized microarray chips were scanned with Affimetrix GeneChip® Scanner 3000 7G. Six scanned data (n = 3 for each of WKY and SHRSP) were normalized by Robust Multiarray Average (RMA) algorithm with Expression Console ver. 1.4 software and then analyzed in Transcriptome Analysis Console software for differentially expressed genes to compare WKY with SHRSP. For upstream analysis of MuRF1, the differentially expressed gene data obtained were cut off by fold change (≥ 2.0 and ≤ -2.0) and *p* value (< 0.05), and then further analyzed with Ingenuity Pathway Analysis (IPA) (QIAGEN). Candidate molecules for MuRF1 up-regulators were determined by calculating the activation z-score (≥ 2.0) and *p* value of overlap (< 0.05) with IPA Upstream Analysis.

2.6. Protein expression analysis by western blotting

Soleus and plantaris were homogenized with RIPA buffer (Thermo Fisher Scientific) supplemented with proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA) and phosphatase inhibitor cocktail set IV (Merck, Kenilworth, NJ, USA). The homogenized samples were incubated at 4 °C for 1 h and then centrifuged at 12,000 \times g at 4 °C for 15 min. The lysed material obtained was mixed with 4 \times sample buffer containing 0.25 M Tris-HCl (pH 6.8), 8% (w/v) SDS, 40% (v/v) glycerol, 8% (v/v) β -mercaptoethanol, and 0.04% (w/v) bromophenol

blue and incubated at 95 °C for 10 min. The samples were separated by SDS-PAGE using a 10% or 15% polyacrylamide gel (5%–20% gradient gel for ubiquitin) and electro-transferred to a polyvinylidene fluoride (PVDF) membrane (Merck-Millipore, Billerica, MA, USA). Western blotting was then performed using antibodies against MuRF1, atrogin-1, ubiquitin, and GAPDH, and then signals were detected using a LAS-4010 instrument (GE Healthcare Life Sciences). The signal intensity was analyzed using the Image J software (National Institute of Health, Bethesda, MD, USA) and normalized to that of GAPDH.

2.7. Statistical analysis

Statistical analysis was carried out with R software (R Foundation for Statistical Computing, Vienna, Austria, <https://www.r-project.org/>). First, the data obtained were analyzed using the Shapiro-Wilk normality test for decision of parametric or non-parametric analyses. For the parametric analysis, F test was further performed for decision of using Student's or Welch's *t*-test. For the non-parametric analysis, Mann-Whitney *U* test was used. *p* < 0.05 was considered a statistically significant difference.

3. Results

3.1. Soleus and gastrocnemius, not plantaris and tibialis anterior muscles, of SHRSP are significantly smaller than those of WKY

Blood pressure of SHRSP was significantly higher than that of WKY (Table 1). In contrast to blood pressure, body weight of SHRSP was significantly lower than that of WKY. We previously showed that blood pressure and body weight showed an inverse correlation between WKY, SHR, and SHRSP [15]. Body and tibial lengths were also significantly different between WKY and SHRSP, although the differences were only slight compared with body weight. Heart weight was not significantly different between the two rats, but heart/tibial length of SHRSP was significantly larger than that of WKY. We also measured the weights of 4 skeletal muscles in the lower leg, soleus, gastrocnemius, plantaris, and tibialis anterior. The skeletal muscle weight of most muscles examined was significantly different between the two rats, except for plantaris muscle. Furthermore, the muscle weights corrected by tibial length showed that soleus and gastrocnemius were significantly different, indicating that muscles consisting of a relatively high amount of slow muscle fiber were affected.

Table 1

Basic characteristics of 12-week-old Wister-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP).

	WKY (n = 6)	SHRSP (n = 6)	<i>p</i> value
Blood pressure (mmHg)	135.9 ± 5.2	240.6 ± 10.7	1.04E-09
Body weight (g)	355.3 ± 19.9	287.7 ± 18.1	1.07E-04
Height (cm)	22.2 ± 0.7	21.0 ± 0.7	0.020
Tibial length (cm)	4.08 ± 0.06	3.79 ± 0.10	0.004
Heart (g)	1.214 ± 0.074	1.274 ± 0.087	0.226
Skeletal muscles (g)			
Soleus	0.119 ± 0.010	0.087 ± 0.008	8.77E-05
Gastrocnemius	1.644 ± 0.105	1.287 ± 0.051	0.002
Plantaris	0.262 ± 0.027	0.242 ± 0.019	0.156
Tibialis anterior	0.574 ± 0.031	0.508 ± 0.029	0.004
Heart/Tibial length (g·cm ⁻¹)	0.298 ± 0.017	0.336 ± 0.027	0.015
Skeletal muscles/Tibia length (g·cm ⁻¹)			
Soleus	0.029 ± 0.003	0.023 ± 0.002	8.80E-04
Gastrocnemius	0.403 ± 0.027	0.340 ± 0.016	0.008
Plantaris	0.064 ± 0.006	0.064 ± 0.006	0.893
Tibialis anterior	0.141 ± 0.008	0.134 ± 0.009	0.182

Values are presented as mean ± S.D. *p* values of WKY vs SHRSP by Student's *t*-test or Man-Whitney *U* test.

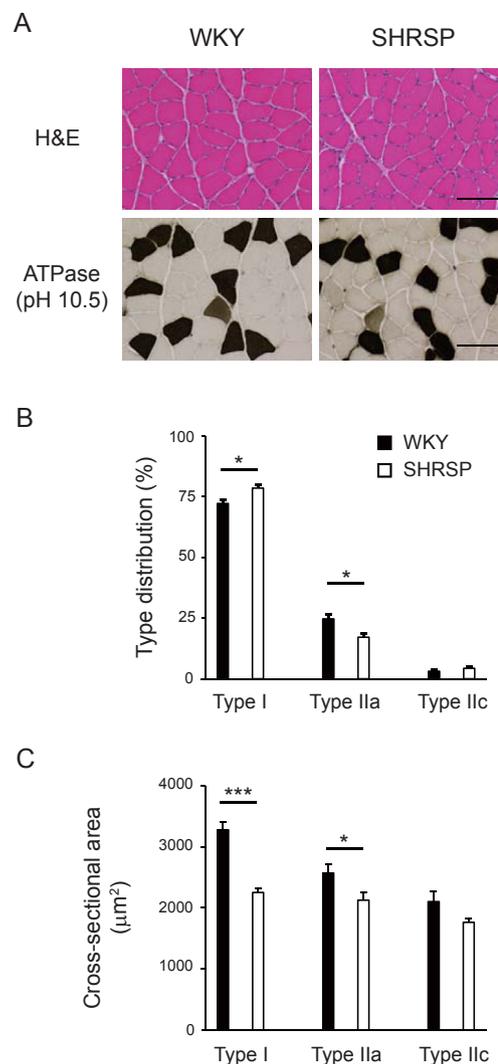


Fig. 1. Histopathological analysis of soleus muscle.

(A) Representative images of hematoxylin-eosin staining and ATPase staining (pH 10.5). Scale bar: 50 μm. (B) Type distribution in soleus muscle. ATPase staining at pH 10.5 was defined in types I (light), IIa (dark), and IIc (light dark). (C) Cross-sectional area by fiber type. **p* < 0.05, ****p* < 0.001.

3.2. Type I fiber in SHRSP showed the most significant hypotrophy among the fiber types compared with WKY

To assess the difference in myofibril in soleus between WKY and SHRSP in addition to weight/tibial length ratio, we first evaluated the histopathological findings by H&E staining (Fig. 1A). Necrosis and regeneration of muscle fibers were hardly observed and were not significantly different between both rats (Supplementary Fig. 1). Inflammation was determined by immunostaining against leukocytes with anti-CD45 antibody (Supplementary Fig. 1). In soleus, WKY had a significantly higher number of leukocytes than SHRSP. However, the soleus of WKY was significantly heavier than that of SHRSP (Table 1). Therefore, there was no significant difference in the leukocytes corrected by the soleus weight between WKY and SHRSP (data not shown). In contrast, there was no significant difference in the number of leukocytes in plantaris between WKY and SHRSP, which also showed no significant difference in weight (Table 1). These results indicate that necrosis, regeneration, and inflammation are not related to slow muscle hypotrophy in SHRSP. Next, to clarify the type distribution in soleus (types I, IIa, and IIc, but not IIb), ATPase staining was carried out at pH 10.5 (Fig. 1A). The ratio of the type I fiber was significantly higher in

SHRSP than in WKY, and vice versa in the ratio of type IIa. The ratio of type IIc was not significantly different (Fig. 1B). We also measured the cross-sectional area by fiber type. Although the cross-sectional area of types I and IIa, but not type IIc, was significantly smaller in SHRSP than in WKY, the degree of the area difference between WKY and SHRSP was much larger in type I fiber than in type IIa fiber (Fig. 1C). The result well reflects the difference in skeletal muscle/tibial length among the 4 skeletal muscles (Table 1).

3.3. Microarray and protein expression analyses showed that muscle-specific ubiquitin ligase, MuRF1, was highly expressed in soleus of SHRSP

To analyze the difference between WKY and SHRSP in more detail, gene expression analysis in soleus was carried out with microarray. First, we focused on the expression of the genes involved in muscle protein degradation, calpain, lysosomal (cathepsins), and ubiquitin-proteasome systems. There was no gene expression difference in calpains (data not shown). For cathepsins, only cathepsin H had a significant difference, showing higher expression in WKY (fold change vs SHRSP; 2.08, $p = 0.032$). These results indicate that calpain and lysosomal systems are not involved in muscle hypotrophy in SHRSP. For the ubiquitin-proteasome system, muscle-specific ubiquitin ligase, MuRF1 (fold change vs WKY; 4.49, $p = 0.003$), but not atrogin-1 (fold change vs WKY; 1.04, $p = 0.830$), was significantly highly expressed in SHRSP. To confirm this gene expression, western blot analysis was carried out for MuRF1 and atrogin-1 (Fig. 2A and B). The result showed that MuRF1, but not atrogin-1, had a significantly higher expression in soleus of SHRSP, which corresponds to gene expression although the fold change is lower in the protein expression level than in the gene expression level (1.75-fold protein expression in SHRSP). In contrast, there was no difference in the MuRF1 and atrogin-1 expression in plantaris (Fig. 2A and B). Furthermore, the effect of ubiquitination by MuRF1 was assessed by western blot (Fig. 2C). Total ubiquitinated proteins in the soleus had an increasing tendency in SHRSP but were not significantly different between WKY and SHRSP. However, ubiquitinated proteins at ~100 kDa were significantly higher in SHRSP (Fig. 2D). In contrast, ubiquitinated proteins in plantaris showed a decreasing tendency in SHRSP, but no significant difference was found between total and ~100-kDa ubiquitinated proteins (Fig. 2D), indicating that the higher expression of MuRF1 in the soleus of SHRSP may be partially involved in higher ubiquitination, especially ~100-kDa proteins.

3.4. Tumor necrosis factor is a candidate for MuRF1 overexpression in SHRSP

Further analysis of differential gene expression between WKY and SHRSP was carried out by IPA. The data were cut off by fold change (≥ 2.0 and ≤ -2.0) and p value (< 0.05), resulting in 259 and 142 genes up-regulated and down-regulated, respectively, in SHRSP, compared to WKY. These genes are listed in Supplementary Table 2, and the clustering analysis is shown in Supplementary Fig. 2. The genes obtained were applied for IPA. Three predicted upstream regulators involved in MuRF1 expression were predicted by a significant p value ($p < 0.001$) and z -score (≥ 2.0) (Table 2). The most likely upstream regulator of the three candidates was tumor necrosis factor (TNF) and then expression of TNF-related receptors in both rats were checked with microarray data. The result showed that two receptors, TWEAKR (fold change vs WKY; 4.52, $p = 0.001$), known as TNF receptor superfamily 12A (TNFRSF12A) and TNF receptor superfamily 22 (TNFRSF22) (fold change vs WKY; 2.89, $p = 0.004$), were significantly highly expressed in SHRSP.

3.5. TWEAKR is physiologically expressed in type II fiber, but its ectopic expression in type I fiber of SHRSP was observed

TWEAKR expression in soleus was focused because Mittal et al. [17] showed that TWEAK expression in skeletal muscle is involved in MuRF1 overexpression, not atrogin-1, which well corresponds to our result (Fig. 2A and B). In contrast, further experiment with TNFRSF22 was not carried out, because the relationship between TNFRSF22 and MuRF1 is unknown. First, we analyzed TWEAKR expression in soleus by western blot (Fig. 3A). The result showed that TWEAKR in soleus had an increasing tendency in SHRSP, but this was not significantly different between WKY and SHRSP. Next, fluorescent immunostaining was carried out (Fig. 3B). It has been reported that TWEAKR staining was partially observed in plasma membrane, but substantially in cytoplasm [18]. Therefore, we evaluated TWEAKR staining in cytoplasm. The result showed that TWEAKR was stained only in type II fiber in WKY, indicating that TWEAKR is a type II fiber-specific receptor in the skeletal muscle. This observation was confirmed with plantaris (Supplementary Fig. 3). In contrast, in addition to type II fiber staining, type I fibers stained were observed in SHRSP, although type II fiber had a higher intensity than type I fiber. These results suggest that ectopic expression of TWEAKR in type I fiber is most likely involved in slow muscle-specific hypotrophy in SHRSP.

4. Discussion

In the present study, we evaluated the skeletal muscle difference between normotensive rats, WKY, and stroke-prone hypertensive rats, SHRSP, by performing histological, gene expression, and protein expression analyses. Our main findings in SHRSP are as follows: (i) skeletal muscles containing a relatively high amount of type I muscle fiber had significantly lower muscle weight/tibial length, (ii) the hypotrophic degree of cross-sectional area of type I fiber in soleus was markedly greater compared with that of type II fibers, (iii) MuRF1, muscle specific ubiquitin ligase, was expressed higher at mRNA and protein levels in soleus, but not in plantaris, and partially up-regulated ubiquitination, and (iv) TWEAKR, which was physiologically expressed in type II fiber, was ectopically expressed in type I fiber.

Interestingly, not all muscles tested in this study had a significant difference in skeletal muscle weight/tibial length between WKY and SHRSP. This difference is distinguished by the ratio of type I muscle fiber. Plantaris and tibialis anterior muscles with no significant difference observed are predominant type II muscle fibers. In contrast, soleus is mainly composed of type I muscle fiber. Gastrocnemius is known to be predominantly consisting of type II muscle fiber, but it contains a relatively higher amount of type I muscle fiber compared to plantaris and tibialis anterior muscles. These results were confirmed by the histological analysis showing that the difference in cross-sectional area in type I fibers between WKY and SHRSP is much larger than that of type II fibers (Fig. 1C). We also observed the difference in type distribution of I and IIa between WKY and SHRSP. This observation for type distribution indicates the possibility that, in SHRSP, i) lowering the fiber size of type I was covered by the number or ii) aging was accelerated. Similar to atrophy by microgravity [19] and other atrophic conditions [20], the type I muscle fiber was more effective than the type II muscle fiber, consistent with our result, whereas type distribution usually changes from type I to type II under the conditions. Aging causes an increase in type I fiber ratio [16], but it decreases type II fiber size [21]. Therefore, it is considered that the skeletal muscle hypotrophy in SHRSP may be due to hypertension.

The balance between protein synthesis and degradation is important for skeletal muscle homeostasis. In this study, we focused on the proteins for the degradation pathway, because protein degradation is promoted on a long-term basis compared to protein synthesis [22]. It may be difficult to explain that MuRF1 alone is involved in skeletal muscle hypotrophy by ubiquitination. However, MuRF1 is mainly

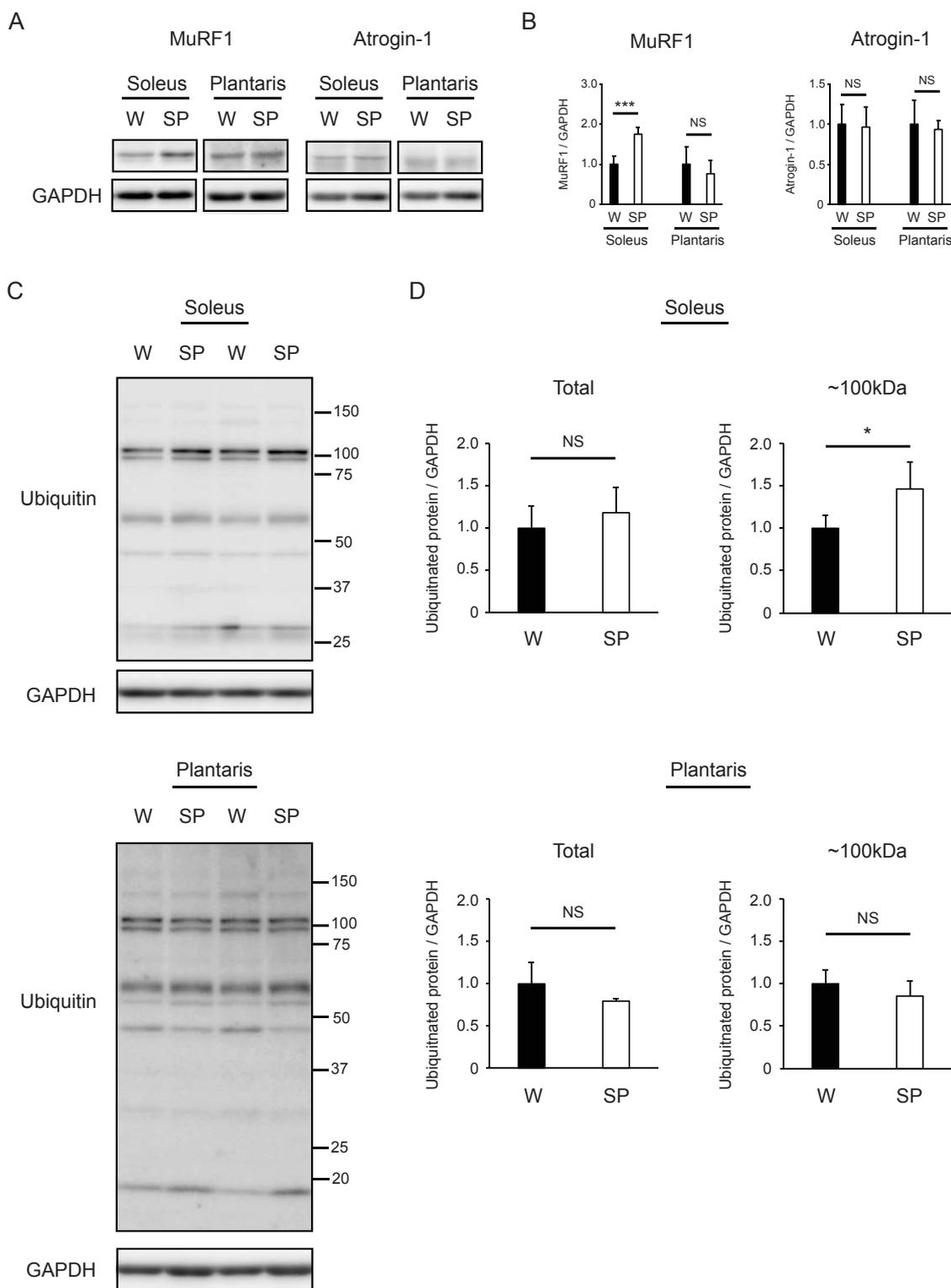


Fig. 2. Expression analysis of muscle MuRF1 and Atrogin-1.

(A) Western blot of soleus and plantaris for MuRF1 and atrogin-1 (n = 6). W: WKY, SP: SHRSP. (B) Densitometric analysis of MuRF1 and atrogin-1. Data are presented as mean + SD, and the mean value of WKY is expressed as 1.0. ***p < 0.001; NS, no significance. (C) Western blots of soleus and plantaris for ubiquitinated proteins (n = 6). (D) Densitometric analysis of ubiquitinated proteins from soleus and plantaris. Data are presented as mean + SD, and the mean value of WKY is expressed as 1.0. *p < 0.05; NS, no significance.

expressed in type II muscle fibers and plays a role for type II fiber maintenance [23]. Thus, increase in MuRF1 expression in soleus may cause more specific effects on type I fiber hypotrophy. Analysis of MuRF1 up-regulator by IPA predicted TNF as a candidate. Further analysis for TNF-related receptors showed that TWEAKR is the most

likely MuRF1 up-regulator. Although there was no significant difference in the TWEAKR protein expression level in soleus between the two rats (Fig. 3A), immunostaining of TWEAKR clarified that both rats had different staining patterns, as shown by the stained type II fibers in WKY and both stained type I and II fibers in SHRSP (Fig. 3B). These

Table 2
Predicted upstream regulator for increasing MuRF1 expression in stroke-prone spontaneously hypertensive rats.

Upstream	Molecule type	Predicted activation	Activation z-score	p value of overlap
Tumor necrosis factor	cytokine	activation	2.290	4.06E-16
lipopolysaccharide	chemical drug	activation	2.556	1.52E-15
dexamethasone	chemical drug	activation	2.320	3.54E-13

results indicate that physiologically specific expression of TWEAKR in type II fiber shown in this study may play a role in type II fiber maintenance by increasing MuRF1 expression as previously indicated [23]. Moreover, muscle-specific TWEAK transgenic mouse shows that atrophy by TWEAK is restricted predominantly in type II fiber and increases MuRF1 expression, but not atrogen-1 expression [17]. This result well corresponds to our result, showing that TWEAKR is a type II fiber-specific receptor in skeletal muscle and MuRF1, not atrogen-1, expression was increased. Thus, ectopic TWEAKR expression in type I fiber is most likely involved in slow muscle-specific hypotrophy in SHRSP. Furthermore, it can be explained by offset that protein expression level of TWEAKR had an increasing tendency in SHRSP, but it was not significantly different from that of WKY. Increasing and decreasing factors for TWEAKR expression in SHRSP are follows: 1) type I fiber of SHRSP increased the rate and ectopic TWEAKR expression than that of WKY (increasing factor); and 2) in contrast, type II fiber, which

mainly expresses TWEAKR, in SHRSP decreased the rate (~7% decrease) compared with that in WKY (decreasing factor).

In the present study, however, we were unable to determine how TWEAKR expression physiologically regulates and what is the regulator(s) accelerating TWEAKR expression in type I fiber. A further study is needed to elucidate regulator(s) for TWEAKR expression in type I fiber to clarify skeletal muscle hypotrophy and the relationship between skeletal muscle hypotrophy and hypertension-related diseases for verification of a therapeutic target.

5. Conclusion

We demonstrated that TWEAKR is a type II fiber-specific receptor in skeletal muscle and its ectopic expression in type I fiber may be related to MuRF1 overexpression in SHRSP soleus (slow muscle), but not in plantaris (fast muscle), thereby causing slow muscle-specific hypotrophy. These findings suggest the possibility that blocking the TWEAKR-MuRF1 pathway could improve slow muscle hypotrophy and be one of the useful therapeutic targets for hypertension and hypertension-related diseases by fixing the skeletal muscle mass and then improving of probable myokine disturbance in slow muscle.

Declaration of competing interest

The authors declare that there are no competing interests.

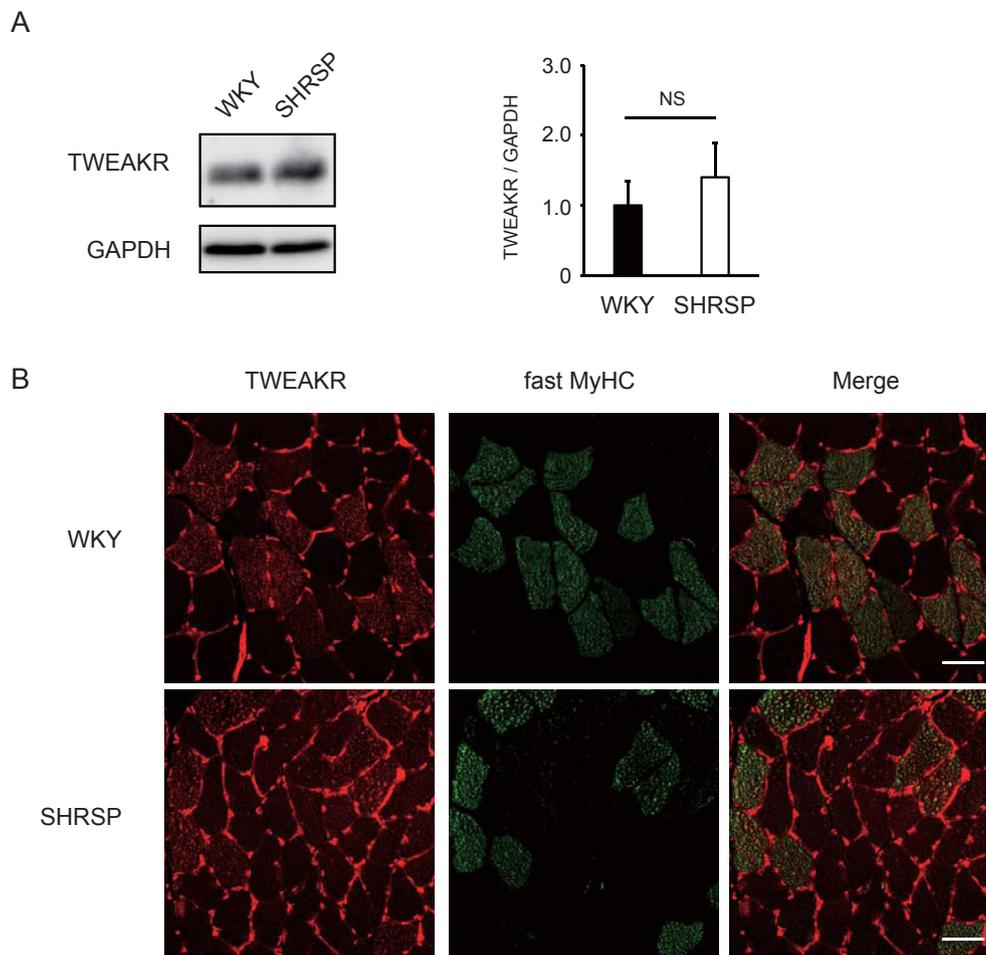


Fig. 3. Expression analysis of TWEAKR.

(A) Western blot and densitometric analysis for TWEAKR in soleus ($n = 6$). Data are presented as mean + SD, and the mean value of WKY is expressed as 1.0. NS, no significance. (B) Immunofluorescence image of soleus. Red, TWEAKR; Green, fast myosin heavy chain (fast MyHC). Scale bar: 50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2019.116919>.

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