

# Aquaporin 4 Expression Level Is Decreased in Skeletal Muscles with Aging

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Skeletal muscle is a tissue that contains abundant water. However, by aging a decrease in muscle water content is induced in skeletal muscles, which is one of major age-related alterations in skeletal muscles as common as muscle atrophy. Selective water channel aquaporin 4 (AQP4) is one of major water transport networks in the skeletal muscles. However, the effects of aging on water transport via AQP4 in skeletal muscles remain unclear. Thus, the current study investigated the change of the expression level of AQP4 in the aged skeletal muscles. Eight-week-old (the young group) and 2-year-old (the old group) female Fischer 344 rats were used in this study (n = 6/group). In skeletal muscles of each group, the expression levels of some target proteins were quantified by Western blot analysis. As a result, the relative muscle weight in the old group was significantly decreased, compared with that in the young group ( $p < 0.05$ ). The decline in the muscle water content was accompanied by the decrease in expression of AQP4 in the aged skeletal muscles ( $p < 0.05$ , respectively). Moreover, the expression of transient receptor potential vanilloid 4, which synergistically regulates the osmolality together with AQP4, was significantly reduced in the aged skeletal muscles ( $p < 0.05$ ). Therefore, the current study suggested that water transport abilities via AQP4 may decrease in the aged skeletal muscles, and thereby may be involved in age-related loss of muscle water content.

## INTRODUCTION

Skeletal muscles are intrinsically abundant in water, which accounts for approximately 76% of the muscle mass [1]. Intracellular water is involved in the regulation of several cell functions, such as cell volume, osmotic pressure and chemical catalyst; therefore, the maintenance of water homeostasis is physiologically important in living organisms [2]. Moreover, intracellular water plays an important role in regulating protein synthesis [3, 4]. On the other hand, the decrease in muscle water content is markedly induced by aging, which is also one of the typical age-induced alterations in skeletal muscles as well as the loss of muscle mass, a decline in muscle strength [5–7].

In the skeletal muscles, the major regulatory factor of water transport is a selective water channel aquaporin 4 (AQP4), which is expressed on the plasma membrane of myofibers [8, 9]. Water transport between AQP4 expressed on the myofiber and aquaporin 1 (AQP1) expressed on the capillaries comprises one of the major water transport networks in skeletal muscles [9, 10]. In general, AQP4 plays important roles in regulating osmotic pressure and energy metabolism in the skeletal muscles [9, 11, 12]. It is also thought that the interaction with transient receptor potential isoform 4 (TRPV4) plays important role to regulate the cell volume and calcium homeostasis, i.e., a water influx via AQP4 is followed by a calcium influx via the opened TRPV4 channel, and then a water efflux via AQP4 plays role in modulating the cell volume and osmotic balance [13–16]. Moreover, it was recently reported that AQP4 is involved in the regulation of morphological changes of skeletal muscles such as overload-induced muscle hypertrophy and disuse-induced muscle atrophy [17, 18]. In this way, AQP4 plays important roles to regulate homeostasis of skeletal muscles such as the regulation of water transport and morphological changes. However, the effects of aging on the characteristics of AQP4 in skeletal muscles remains unclear while the decrease in muscle water content is shown in aged skeletal muscles.

The current study investigated the effects of aging on the expression of AQP4 in the skeletal muscles. In addition, this study also demonstrated the age-induced changes of the expression of AQP1 and TRPV4, which interact and function as regulatory factors of water transport together with AQP4 in skeletal muscles.

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## MATERIALS AND METHODS

### Experimental design and surgical procedure

Eight-week-old and 2-year-old female Fischer 344 rats were used in this study. Eight-week-old rats composed the young group and 2-year-old rats composed the old group ( $n = 6/\text{group}$ ). Two or three rats were housed in one cage at 22°C with a 12-h light/dark cycle, and were provided food and water *ad libitum*. At the time of sacrifice, the rats were anesthetized under pentobarbital sodium anesthesia (60 mg kg<sup>-1</sup> i.p.) and inhalation anesthesia with 0.7% isoflurane, and the tibialis anterior muscles (TA) and soleus muscles (SOL) were removed. All experiments were carried out following approval from the Ethics Committee on Life Sciences of Osaka Institute of Technology (Approval No. 2014-20-8).

### Muscle water content

Wet weights of muscle samples were measured. Then, each sample was completely dried for 24 h at 100°C and the dry weight of each sample was measured. The relative muscle water content of each sample was calculated using the following formula:  $([\text{wet weight} - \text{dry weight}] \times \text{wet weight}^{-1}) \times 100$ .

### Western blot analysis

Sample preparation and Western blot analysis were performed according to the protocol described in a previous study [17]. In brief, the muscle samples were homogenized in 300 µl of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) (Sigma-Aldrich, St Louis, MO) containing 1x Protease Inhibitor Cocktail (Roche Diagnostic, Dubai, UAE). After the homogenate samples were centrifuged at 15000 × g for 15 min at 4°C, the supernatants were collected. We used the BCA protein assay kit (Thermo scientific, Rockford, IL) to measure the protein concentration.

The homogenate samples (10 µg of protein) were resolved by SDS-PAGE using either a 12.5% polyacrylamide gel for AQP4, AQP1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or a 7.5% gel for TRPV4, and then transferred onto polyvinylidene difluoride membranes (ATTO, Tokyo, Japan) at 153 mA for 30 min. After transfer, to block any nonspecific immunoreactivity, the membranes were incubated with 20 mM Tris-buffered saline (TBS) (pH 7.6) containing 8% skim milk and 0.1% Tween-20 for 1 h at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies. The primary antibodies were diluted in 20 mM TBS containing 8% skim milk and 0.1% Tween-20. The primary antibodies used in the current study were as follows: goat polyclonal anti-AQP4 (sc-9888, Santa Cruz biotechnology, Santa Cruz, CA, diluted 1:300), mouse monoclonal anti-GAPDH antibody (internal control) (2118, Cell Signaling Technology, Danvers, MA, diluted 1:2000), rabbit polyclonal anti-TRPV4 antibody (ACC-034, Alomone Labs, Jerusalem, Israel, diluted 1:2000) and rabbit monoclonal Anti-AQP1 antibody (ab168387, Abcam, Cambridge, UK, diluted 1:3000). All of the primary antibodies used in the current study has been recognized as the species cross-reactivity to rats. In addition, these primary antibodies used in the current study were reported to have been worked appropriately for the detection of target proteins in the skeletal muscles in mammals by previous studies [9, 12, 18, 19]. Then, they were washed in 20 mM TBS containing 8% skim milk and 0.1% Tween-20, and incubated for 1 h at room temperature with secondary antibodies. The secondary antibodies used in the current study were as follows: biotinylated anti-goat IgG (AP180B, Merck Millipore, Darmstadt, Germany, diluted 1:10000), biotinylated anti-rabbit IgG (65-6140, Invitrogen, Camarillo, CA, diluted 1:10000) and biotinylated anti-mouse IgG (BA-2001, Vector Laboratories, Burlingame, CA, diluted 1:10000). They were washed in 20 mM TBS containing 0.1% Tween-20, and incubated for 1 h at room temperature with TBS containing 0.1% Tween-20 and peroxidase conjugated streptavidin horseradish (GE Healthcare, Buckinghamshire, UK). Lastly, immunoreactivity was detected by chemiluminescence using ImmunoStar Zeta (Wako, Osaka, Japan). The bands were quantified by densitometric analysis using ImageJ software (ver.1.48, <http://rsb.info.nih.gov/ij/>). The mean value for the young group on each immunoblot, expressed relative to GAPDH as an internal control, was adjusted to equal 1.0, and each sample value was expressed relative to the adjusted mean value for the young group.

### Statistical analysis

All data are presented as means ± SD and were analyzed using the StatView statistical-analysis program (SAS institute Inc., Cary, NC, USA). Normality was confirmed using the Shapiro-Wilk test. Based on the distribution of the data, each data was tested by the Student's t-test or Mann-Whitney U-test. Differences were considered significant at a confidence level of 0.05.

## RESULTS

**Effects of aging on body weights, muscle wet weights, relative weights and water content of TA and SOL**

Body weights, muscle wet weights and relative muscle weights of each group were indicated in Table I. Body weights of the young and old groups were  $137.6 \pm 3.6$  g and  $356.6 \pm 39.0$  g ( $p < 0.05$ ). Muscle wet weights of TA were respectively  $230.00 \pm 13.20$  mg and  $379.71 \pm 26.14$  mg in the young and old groups ( $p < 0.05$ ) and that of SOL were respectively  $54.29 \pm 2.81$  mg and  $82.29 \pm 4.21$  mg in the young and old groups ( $p < 0.05$ ). Moreover, to assess the net effects of aging on the weights of TA and SOL, we calculated the relative muscle weights per 100 g body weights in each group. As a results, in TA the relative weight in the old group ( $107.23 \pm 9.88$  mg/100 g body weight) was significantly lower than that in the young group ( $167.11 \pm 6.55$  mg/100 g body weight) ( $p < 0.05$ ). Similarly, in SOL the relative weight in the old group ( $23.28 \pm 2.48$  mg/100 g body weight) was also significantly lower than that in the young group ( $39.45 \pm 1.53$  mg/100 g body weight) ( $p < 0.05$ ).

Furthermore, the current study measured the water content in TA and SOL in the young and old groups. As shown in Fig. 1A, the water content in TA in the old group was significantly lower than that in the young group ( $p < 0.05$ ). Similarly, the water content in SOL in the old group was significantly lower than that in the young group ( $p < 0.05$ ) (Fig. 1B). Therefore, both the relative weight and water content significantly decreased in TA and SOL with age.

Table I. Effects of aging on body weight, muscle wet weight and relative muscle weight in tibialis anterior muscles and soleus muscles

	young	old
Body weight (g)	$137.57 \pm 3.55$	$356.57 \pm 39.02^*$
TA		
muscle wet weight (mg)	$230.00 \pm 13.20$	$379.71 \pm 26.14^*$
Relative muscle weight (mg/100 g body weight)	$167.11 \pm 6.55$	$107.23 \pm 9.88^*$
SOL		
muscle wet weight (mg)	$54.29 \pm 2.81$	$82.29 \pm 4.21^*$
Relative muscle weight (mg/100 g body weight)	$39.45 \pm 1.53$	$23.28 \pm 2.48^*$

TA: tibialis anterior muscles, SOL: soleus muscles, young: age 8 weeks, old: age 2 years. Values are mean  $\pm$  S.D.

Relative muscle weights are presented the relative muscle weights per 100 g body weight.

\* $p < 0.05$ , compared with the young group (SOL, Mann-Whitney U-test).

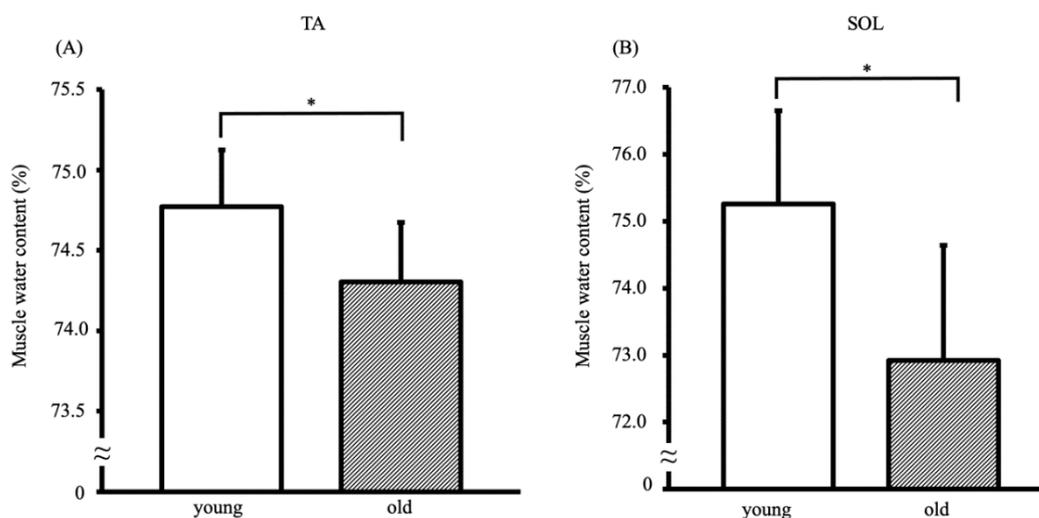


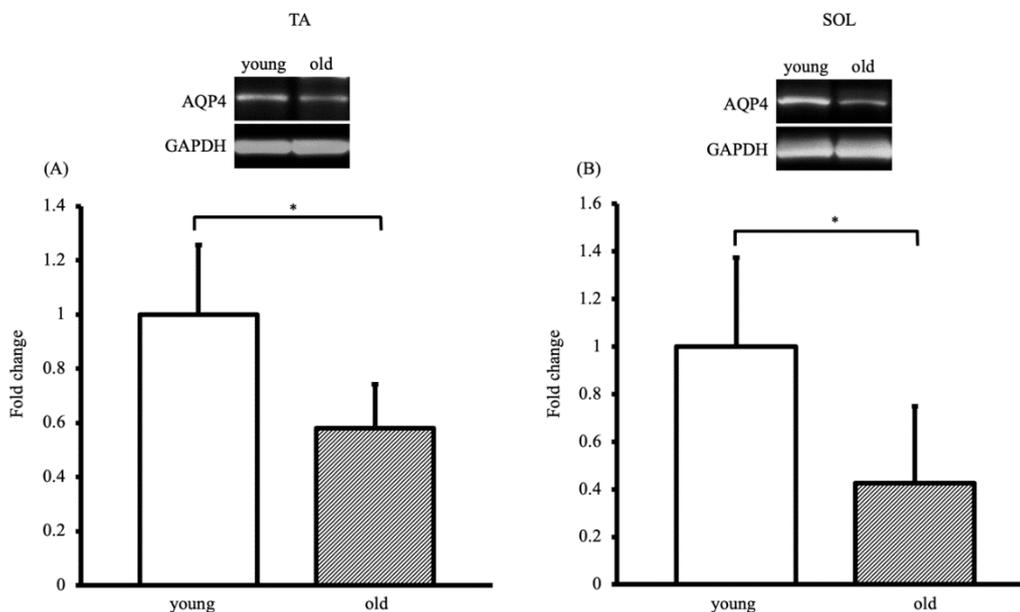
Fig. 1. Decrease in muscle water content in tibialis anterior muscles and soleus muscles in old rats.

TA: tibialis anterior muscles, SOL: soleus muscles, young: aged 8 weeks, old: aged 2 years.

Values are the mean  $\pm$  SD. \* $p < 0.05$ .

### Effects of aging on AQP4 expression in TA and SOL

We performed Western blot analysis using the AQP4 antibody to examine the effects of aging on AQP4 expression in TA and SOL. As shown in Fig. 2A, AQP4 expression in TA of the old group was significantly lower than that of the young group ( $p < 0.05$ ). Similarly, in SOL, the expression of AQP4 in the old group was significantly lower than that in the young group ( $p < 0.05$ ) (Fig. 2B). Therefore, the expression of AQP4 was significantly reduced in both TA and SOL by aging.



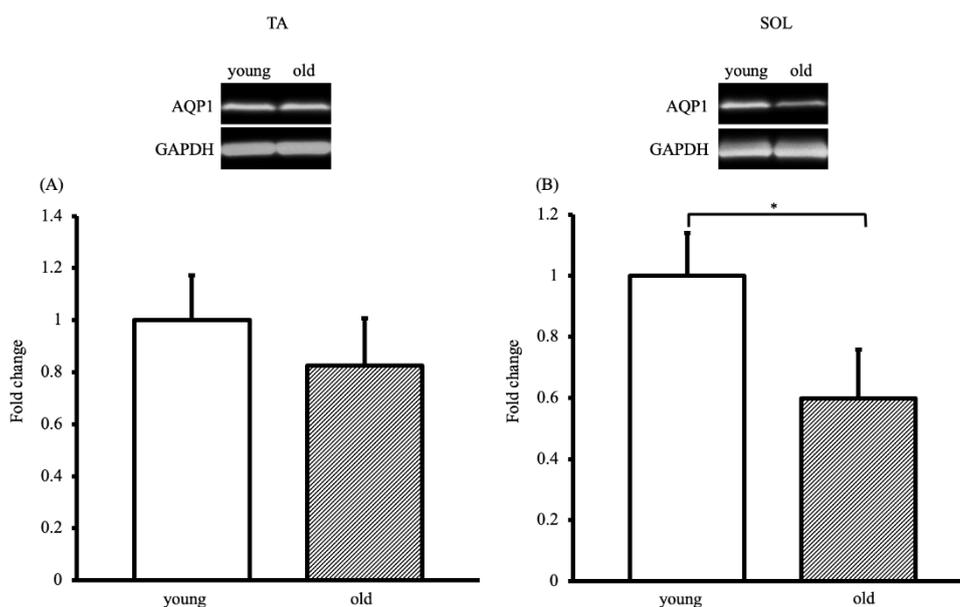
**Fig.2.** Decrease in AQP4 expression in tibialis anterior muscles and soleus muscles in old rats.

TA: tibialis anterior muscles, SOL: soleus muscles, young: aged 8 weeks, old: aged 2 years.

AQP4 protein expression levels, normalized by the GAPDH protein expression level, were calculated by densitometric analysis. Values are the mean  $\pm$  SD. Fold changes were expressed relative to the levels observed in the young group. \* $p < 0.05$ .

### Differences in AQP1 expression in TA and SOL with age

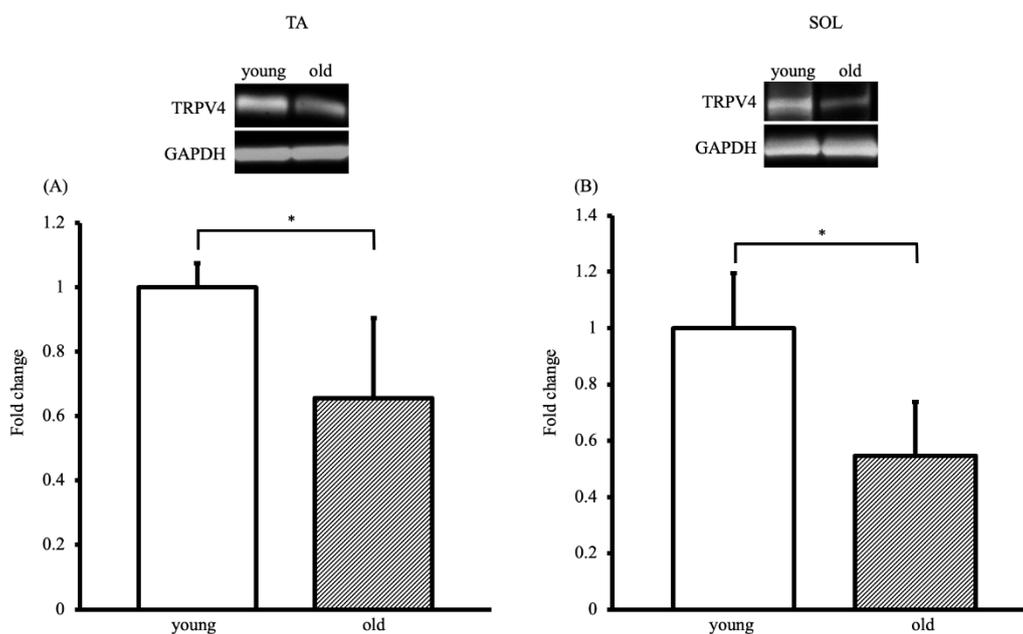
To evaluate the changes in AQP1 expression in TA and SOL with age, we examined AQP1 expression by Western blot analysis. As a result, in TA, there was no significant difference between the young and old groups (Fig. 3A). On the other hand, in SOL, the expression of AQP1 in the old group was significantly lower than that in the young group ( $p < 0.05$ ) (Fig. 3B). Therefore, the changes in expression of AQP1 with age differed between TA and SOL.



**Fig.3.** Effect of aging on AQP1 expression in tibialis anterior muscles and soleus muscles in old rats. TA: tibialis anterior muscles, SOL: soleus muscles, young: aged 8 weeks, old: aged 2 years. AQP1 protein expression levels, normalized by the GAPDH protein expression level, were calculated by densitometric analysis. Values are the mean  $\pm$  SD. Fold changes were expressed relative to the levels observed in the young group. \* $p < 0.05$ .

#### Decrease in TRPV4 expression in TA and SOL with age

We investigated changes in TRPV4 expression in TA and SOL due to aging using Western blot analysis. In TA, the expression of TRPV4 in the old group was significantly lower than that in the young group ( $p < 0.05$ ) (Fig. 4A). In SOL, the expression of TRPV4 in the old group was also significantly lower than that in the young group ( $p < 0.05$ ) (Fig. 4B). Therefore, the expression of TRPV 4 significantly decreased in both TA and SOL with age.

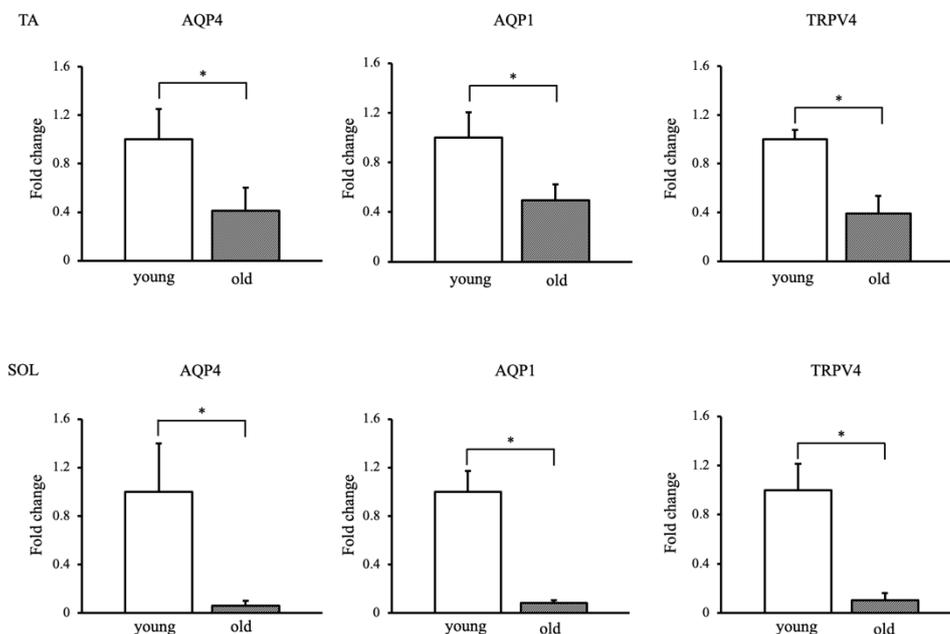


**Fig. 4.** Decrease in TRPV4 expression in tibialis anterior muscles and soleus muscles in old rats. TA: tibialis anterior muscles, SOL: soleus muscles, young: aged 8 weeks, old: aged 2 years. TRPV4 protein expression levels, normalized by the GAPDH protein expression level, were calculated by densitometric analysis. Values are the mean  $\pm$  SD. Fold changes were expressed relative to the levels observed in the young group. \* $p < 0.05$  (TA, Mann-Whitney U-test).

## AQP4 DECREASE IN AGED SKELETAL MUSCLES

### Change of AQP4, AQP1 and TRPV4 expression level per muscle water volume in TA and SOL with age

We calculated the AQP4, AQP1 and TRPV4 expression level divided by the muscle water volume in TA and SOL, respectively. As a result, in both TA and SOL, all parameters in the old group were significantly decreased than that in the young group ( $p < 0.05$ , respectively) (Fig.5).



**Fig.5.** Changes of AQP4, AQP1 and TRPV4 expression level per muscle water volume in tibialis anterior muscles and soleus muscles with age. TA: tibialis anterior muscles, SOL: soleus muscles, young: aged 8 weeks, old: aged 2 years. AQP4, AQP1 and TRPV4 expression level divided by muscle water volume were respectively calculated. Fold changes were expressed relative to the levels observed in the young group. \* $p < 0.05$ .

## DISCUSSION

In the current study, the relative muscles weights in the old group were significantly lower than those in the young group. Previous studies reported the marked induction of muscle atrophy by aging [20, 21]. In the current study, age-induced muscle atrophy was observed in the old group. Moreover, the muscle water content in the old group was significantly lower than that in the young group. It was previously demonstrated that the total body water and muscle water content significantly decrease with age [6, 22, 23]. Therefore, consistent with previous studies, in the current study the decrease in muscle water content in both TA and SOL were considered to be due to aging. Moreover, the intracellular water plays an important role in regulating the cell volume and protein synthesis. Although its detail molecular mechanisms have been poorly understood, it is suggested that changes in mechanical stretch to the cell membrane due to surface tension of water affect the intracellular signal transduction pathway involved in protein synthesis [2, 4, 6]. Therefore, the current study suggested that the decrease in muscle water content may be an important factor in age-related muscle atrophy.

The current study demonstrated that the AQP4 expression level significantly decreased in both aged TA and SOL, and thus the decrease in AQP4 expression level was common aging phenomenon in both fast and slow skeletal muscles as well as loss of muscle water content. In a previous study, water deprivation led to a decrease in AQP4 expression in the skeletal muscles [24]. It is well known that the total body water and muscle water content markedly decrease with age [7, 22, 23]. Indeed, in the current study, the muscle water content in aged skeletal muscles was significantly lower than that in young skeletal muscles. Therefore, there may be relationship between the decrease in body and muscle water content and the decrease in AQP4 expression in skeletal muscles due to aging.

The current results showing the decrease of AQP4 in the aged skeletal muscles were supported by previously reported that AQP4 was down-regulated in the aged skeletal muscles [25]. Therefore, it was suggested that the decrease of AQP4 in the aged skeletal muscles may be attributed to the decline in the transcription levels of AQP4. However, the current study was unable to clarify the molecular mechanism of the decrease in AQP4 expression in skeletal muscles with age. It was previously demonstrated that AQP4 expression is regulated by the innervation status in skeletal muscles, i.e., in skeletal muscles, the AQP4 expression level was markedly reduced by

denervation [17, 18, 26]. The functional activities, cell size and total number in motoneurons innervating myofibers significantly decreased with age, thereby promoting the dysfunction of excitation-contraction coupling and onset of denervation in aged skeletal muscles [5, 27, 28]. Therefore, the current study suggested that age-induced regression of neuromuscular systems functions in the decrease in AQP4 expression in aged skeletal muscles.

The current study demonstrated for the first time that the decreases in muscle water content and AQP4 expression level were common aging-associated phenomenon in both fast and slow muscles. On the other hand, the current study indicated that degrees of the decrease in the relative muscle weights, muscle water content and AQP4 expression level in SOL with aging were slightly higher than that in TA. These results were partly supported by the previous studies that reported that the cellular water volume was involved in the regulation of cell volume [3, 4]. However, in the current study, it was very difficult to correctly explain why these differences in aging response occurred between fast and slow muscles. In the previous study, it was reported that the fast-twitch myofibers had a higher ability to regulate water content in response to the changes of the extracellular environments than in slow-twitch myofibers [29]. So, these differences in ability may possibly influence the results shown in the current study. However, further studies need to elucidate molecular mechanisms that induced the differences in aging responses in muscle water content and AQP4 between fast and slow muscles.

Of note, the expression of TRPV4 in aged skeletal muscles was significantly lower than that in young skeletal muscles. In general, AQP4 interactively regulates the osmotic pressure and cell volume regulation together with TRPV4 [13–16]. Therefore, the abilities to regulate osmotic pressure and cell volume may decrease in aged muscle concomitant with the decrease in AQP4 and TRPV4 with age. The current study cannot elucidate the molecular mechanisms to regulate the decrease in the AQP4 and TRPV4 expression in the skeletal muscles with aging. In the previous study, it was demonstrated that AQP4 and TRPV4 were sensitive to the change of intracellular calcium dynamics in glial cells [30–32]. Therefore, in the current study, the decrease in AQP4 and TRPV4 expression may be possibly incused by the changes in calcium kinetics associated with decreased contractile activity of skeletal muscle with aging. However, even if so, molecular mechanisms regulating the decrease of AQP4 and TRPV4 expression remain unclear, and the further studies need to elucidate the regulatory mechanisms of the expression of AQP4 and TRPV4 in the skeletal muscles with aging.

To our knowledge, no previous studies have reported the effects of aging on the functional properties of AQP4 and TRPV4 in the skeletal, and the current study could not elucidate the functional properties of these proteins in the aged skeletal muscles. Therefore, the further studies need to elucidate the functional changes in the skeletal muscles with aging.

Unlike AQP4 expression in aged skeletal muscles, AQP1 expression in response to aging differed between TA and SOL in the current study. However, it is difficult to explain the difference in AQP1 expression in response to aging between TA and SOL. In general, AQP1 is expressed on the capillaries within the skeletal muscles [15, 16]. The relationship between capillary supply and myofiber size is maintained with age, whereas the capillary supply regresses in skeletal muscles with age [33–36]. Moreover, as the current study did not evaluate the effects of aging on the capillary supply in skeletal muscle, whether there was a difference in capillary supply in response to aging between TA and SOL remains unclear. In the current study, the rates of decline in muscle water content and AQP4 expression in SOL were higher than those in TA. Thus, the hypoactivity of water transport in aged SOL may have progressed further than that in aged TA, which may have been reflected in the differences in AQP1 expression patterns. Further studies are needed to clarify the differences in the effects of aging on the capillary supply and AQP1 expression between fast muscles (TA) and slow muscles (SOL).

According to the results of change of AQP4, AQP1 and TRPV4 expression levels per muscle water volume in TA and SOL with age, it was suggested that the expression levels of AQP4, AQP1, and TRPV4 were reduced more than the reduction in muscle water content in both aged TA and SOL. Therefore, these results were suggested that the decrease of water transport related molecules such as AQP4, AQP1 and TRPV4, may be occurred prior to the decrease of muscle water volume with age. On the other hand, because the current study did not investigate the effect of aging on other factors regulating water transport, whether changes of these factors investigated in the current study directly regulated the changes of muscle water volume with age remain unclear and it was not ruled out the possibility that other factor may modulate the changes of these factors and muscle water volume with age. Therefore, further studies are needed to elucidate how water transport related molecules such as AQP4, AQP1 and TRPV4 are related to the muscle water volume loss with age.

In conclusion, the current study first demonstrated that decrease in AQP4 expression in both TA and SOL by aging, in addition to loss of muscle water content. Therefore, it was suggested that age-related loss of muscle water content may be associated with a decrease in water transport abilities via AQP4 in both aged fast muscles and slow muscles. Moreover, in the current study, muscle atrophy, loss of muscle water content and decrease in AQP4 expression were all identified in the aged skeletal muscles, suggesting that these factors may be related to each other as a phenomenon of aging in skeletal muscles.

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## CONFLICT INTEREST

The authors declare that they have no competing interests.

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