

Aspiration pneumonia induces muscle atrophy in the respiratory, skeletal, and swallowing systems

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Abstract

Background Repetition of the onset of aspiration pneumonia in aged patients is common and causes chronic inflammation. The inflammation induces proinflammatory cytokine production and atrophy in the muscles. The proinflammatory cytokines induce muscle proteolysis by activating calpains and caspase-3, followed by further degradation by the ubiquitin-proteasome system. Autophagy is another pathway of muscle atrophy. However, little is known about the relationship between aspiration pneumonia and muscle. For swallowing muscles, it is not clear whether they produce cytokines. The main objective of this study was to determine whether aspiration pneumonia induces muscle atrophy in the respiratory (the diaphragm), skeletal (the tibialis anterior, TA), and swallowing (the tongue) systems, and their possible mechanisms.

Methods We employed a mouse aspiration pneumonia model and computed tomography (CT) scans of aged pneumonia patients. To induce aspiration pneumonia, mice were inoculated with low dose pepsin and lipopolysaccharide solution intra-nasally 5 days a week. The diaphragm, TA, and tongue were isolated, and total RNA, proteins, and frozen sections were stored. Quantitative real-time polymerase chain reaction determined the expression levels of proinflammatory cytokines, muscle E3 ubiquitin ligases, and autophagy related genes. Western blot analysis determined the activation of the muscle proteolysis pathway. Frozen sections determined the presence of muscle atrophy. CT scans were used to evaluate the muscle atrophy in aged aspiration pneumonia patients.

Results The aspiration challenge enhanced the expression levels of proinflammatory cytokines in the diaphragm, TA, and tongue. Among muscle proteolysis pathways, the aspiration challenge activated caspase-3 in all the three muscles examined, whereas calpains were activated in the diaphragm and the TA but not in the tongue. Activation of the ubiquitin-proteasome system was detected in all the three muscles examined. The aspiration challenge activated autophagy in the TA and the tongue, whereas weak or little activation was detected in the diaphragm. The aspiration challenge resulted in a greater proportion of smaller myofibers than in controls in the diaphragm, TA, and tongue, suggesting muscle atrophy. CT scans clearly showed that aspiration pneumonia was followed by muscle atrophy in aged patients.

Conclusions Aspiration pneumonia induced muscle atrophy in the respiratory, skeletal, and swallowing systems in a preclinical animal model and in human patients. Diaphragmatic atrophy may weaken the force of cough to expectorate sputum or mis-swallowed contents. Skeletal muscle atrophy may cause secondary sarcopenia. The atrophy of swallowing muscles may weaken the swallowing function. Thus, muscle atrophy could become a new therapeutic target of aspiration pneumonia.

Keywords Aspiration pneumonia; Myokine; Sarcopenia; Muscle atrophy; Aged patients

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Introduction

Among pneumonia inpatients aged 70 and older, aspiration pneumonia occurs in 80%, and pneumonia has become the third leading cause of death in Japan.¹ Generally, pneumonia patients are treated according to guidelines, which basically prescribe the application of antibiotics.² Treatments other than antibiotics for aspiration pneumonia are prophylactic, such as by improving cough or swallowing reflexes.^{2–4} The cough reflex is improved by the administration of angiotensin-converting enzyme inhibitor, an anti-hypertensive medicine, and capsaicin, a pungent in red chilli peppers.^{5,6} The swallowing reflex is improved by theophylline, an asthma medicine, and menthol, a mint ingredient.^{7,8}

The number of deaths caused by pneumonia is increasing due to ageing of the population and a new target for treating aspiration pneumonia is needed. Cough is the most potent protective mechanism against aspiration and requires the activation of the inspiratory and expiratory respiratory muscles.⁹ The diaphragm is central among the respiratory muscles.¹⁰ Dysfunction of swallowing physiology is one of the mechanisms that causes aspiration pneumonia.² The tongue plays a pivotal role in swallowing.¹¹ Aged pneumonia patients often have repeated episodes of aspiration pneumonia, which causes chronic in addition to acute inflammation.¹ The inflammation induces muscle atrophy in which a number of cellular mechanisms have been implicated. Chief among these is exposure to proinflammatory cytokines.¹²

Skeletal muscles, such as respiratory muscles and limb muscles, produce cytokines.^{12–15} For the past decade, muscle-derived cytokines and other peptides have been called myokines.¹⁶

Proinflammatory cytokines induce muscle proteolysis by a two-step process, consisting of initial myofibrillar protein cleavage by calpains and/or caspase-3, followed by further degradation by the ubiquitin-proteasome system.^{12,17} Activated calpains and caspase-3 cleave fodrin into specific 145–150 kDa and 120 kDa products, respectively.¹² Two muscle-specific E3 ubiquitin ligases, muscle RING-finger protein 1 (MuRF-1) and atrogin-1, were implicated in inflammation-induced muscle atrophy, such as sepsis.¹⁸ Autophagy has been suggested as another pathway of muscle proteolysis during muscle atrophy in sepsis and intermittent hypoxia.^{19,20}

The original definition of sarcopenia is the loss of skeletal muscle mass and strength that occurs with advancing age.²¹ Recently, the cause of primary sarcopenia was suggested to be ageing itself, and the cause of secondary resulted from triggers such as organ failure or inflammatory diseases. Sarcopenia is a risk factor for dysphagia in aged people.²²

To date, it has not been known whether aspiration pneumonia induces the production of proinflammatory cytokines and atrophy in muscles. For swallowing muscles, as far as

we knew, it has not been clear whether they produce cytokines. The main objective of this study was to determine whether aspiration pneumonia induces muscle atrophy in the respiratory, skeletal, and swallowing systems, and their possible mechanisms. To achieve this objective, we employed a mouse aspiration pneumonia model,¹ and isolated the diaphragm as a respiratory muscle, the tibialis anterior (TA) as a skeletal muscle, and the tongue as a swallowing muscle from control or aspiration-challenged mice every 7 days until Day 28. We also evaluated the muscle size in aged aspiration pneumonia patients using computed tomography (CT) scans.

Materials and methods

Mice

Specific pathogen-free 7- to 10-week-old male C57BL/6 mice (Charles River Japan, Kanagawa, Japan) were housed under barrier conditions. Mice were anaesthetized by intraperitoneal injection of ketamine and xylazine. The Laboratory Animal Committee at Tohoku University approved all experimental procedures.

Mouse aspiration pneumonia model

Mouse aspiration pneumonia was induced as previously described.¹ In brief, phosphate buffered saline (PBS) was thickened to mimic food with 12 mg/mL toromerin® (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Pepsin (2 mg/mL; Sigma, St Louis, MO) was dissolved into the PBS at pH 1.6 adjusted by HCl to mimic gastric juice. Lipopolysaccharide (LPS) (Sigma) was dissolved in the PBS (2.5 mg/mL). We anaesthetized and challenged the mice by intranasal inoculation of 25 µL of pepsin and 20 µL of LPS, respectively, 5 days a week for the indicated periods. The diaphragm, the TA, and the tongue were isolated from the mice every 7 days up to 28 days of aspiration challenge.

Ribonucleic acid (RNA) extraction and quantitative real-time polymerase chain reaction (Q-RT-PCR)

RNA extraction and Q-RT-PCR were performed as previously shown.^{12,15} In brief, total RNA was extracted from muscles using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and purified by the RNeasy minikit (Qiagen, Venlo, The Netherlands). Specific primers were designed to detect the expression of target genes (Table 1). Q-RT-PCR with SYBR™ Green PCR MasterMix (Thermo Fisher, Waltham, MA, USA) was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A dissociation

Table 1 Primer sequences used for Q-RT-PCR analysis

Name	Forward primer	Reverse primer
IL-1 β	5'-GGGCCTCAAAGGAAAAGAATC-3'	5'-TACCAGTTGGGGAAGTCTGC-3'
IL-6	5'-CCGGAGAGGAGACTTCACAG-3'	5'-CAGAATTGCCATTGCACAAC-3'
MCP-1	5'-CCCAATGAGTAGGCTGGAGA-3'	5'-TCTGGACCCATTCTCTTG-3'
MuRF-1	5'-ACCTGCTGGTGGAAAACATC-3'	5'-AGGAGCAAGTAGGCACCTCA-3'
atrogin-1	5'-ATTCTACTGGCAGCAGCA-3'	5'-TCAGCCTCTGCATGATGTTTC-3'
Bnip3	5'-TTCCACTAGCACCTTCTGATGA-3'	5'-GAACACCGCATTACAGAACA-3'
LC3B	5'-CCGGAGCTTTGAACAAAGAGTG-3'	5'-CTTGGTCTTGTCCAGGACGG-3'
Gabarapl1	5'-CAGCTGTATGAGGACAACCAC-3'	5'-CAAGTCCAGGTGCTCCCAT-3'
β -actin	5'-CGACAACGGCTCCGGCATGT-3'	5'-TCTGGCCTCGTACCCACA-3'

MuRF-1, muscle RING-finger protein 1; Bnip3, Bcl2/adenovirus E1B 19 kDa interacting protein 3; LC3B, microtubule-associated protein 1 light chain 3B; Gabarapl1, GABA(A) receptor-associated protein like 1.

curve was analysed for each PCR experiment to assess primer dimer formation or contamination. β -actin was selected as the housekeeping gene, and the relative messenger RNA (mRNA) levels (expressed as fold change compared with the control group) were determined.

Western blotting

Western blotting was performed as previously described.^{12,15} Dilutions and manufacturers of the antibodies were as follows: anti- α -fodrin (1:1000; ENZO, New York, NY, USA), anti-Bnip3 (1:1000; Cell Signalling Technology, Danvers, MA, USA), anti- β -actin (1:5000; Sigma-Aldrich, Saint Louis, MO, USA), anti-mouse IgG (H + L) HRP (1:2500; Promega, Madison, WI, USA), and anti-Rabbit IgG (H + L) HRP (1:2500; Promega). The signals were detected and quantified using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, England) and quantified using ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare).

Histological analysis

We anaesthetized the mice with ketamine and xylazine. Mice were perfused and the muscles (diaphragms, TAs, and tongues) were isolated as described previously.¹ Excised muscles were stored at -80°C . Eight micrometre-thin cryostat sections of TAs and tongues, and 10 μm -thin cryostat sections of diaphragms were stained with haematoxylin and eosin. Images were photographed using an OLYMPUS BX51 microscope (OLYMPUS, Tokyo, Japan) with Lumina Vision software (Mitani Corporation, Fukui, Japan). For analysis of the tissue sections, 500 muscle fibres per sample were measured by Lumina Vision software (Mitani Corporation).

Human muscle quantification

Computed tomography scans were kept in Yahgee Image (FUJIFILM Medical IT Solutions Co, Tokyo, Japan). We quantified the cross-sectional areas of the dorsal muscle group at

the twelfth thoracic vertebral (Th12) level with WeVIEW Z (Hitachi, Tokyo, Japan).²³ CT scans of aspiration pneumonia patients were taken at two time points, before and after the treatment. Aspiration pneumonia was diagnosed as previously shown.^{6,24} In brief, aged pneumonia inpatients 70 years and older with symptoms of dysphagia were assessed for the latency time of the swallowing reflex. For the assessment, a bolus of 1 mL of distilled water was injected into the pharynx through a nasal catheter (8Fr) to stimulate the swallowing reflex. Swallowing was identified by visual observation of the characteristic laryngeal movement. The latency time of the swallowing reflex was measured as the time from the injection to the onset of swallowing. Aged pneumonia patients with a latency time of the swallowing reflex >3 s were diagnosed as aspiration pneumonia. CT scans of eight patients were evaluated. The Human Subjects Institutional Review Board of the Tohoku University Ethics Committee approved the protocol.

Statistical analysis

Values are presented as means \pm SEM. Statistical analysis was performed using ANOVA with the Fisher least significant difference test for multiple comparisons. A *P* value of less than 0.05 was considered significant.¹²

Results

Effects of the aspiration challenge on the production of proinflammatory cytokines in the respiratory, skeletal, and swallowing muscles.

To induce aspiration pneumonia, we challenged mice with low doses of pepsin and LPS 5 days a week.¹ The mRNA expression levels of proinflammatory cytokines (IL-1 β , IL-6, and MCP-1) were determined in the diaphragm and the TA limb muscles every 7 days until Day 28. The expression levels of the cytokines in the diaphragm were greater than those

of controls at all the time points (Figure 1A–C). In the TA, the expression levels of IL-1 β were greater at all time points, IL-6 was greater on Day 21, and MCP-1 was greater on Days 7 and 28 (Figure 1A–C). As far as we know, it has not been clear whether the tongue produces cytokines. Aspiration challenge induced greater expression levels of proinflammatory cytokines after Day 14 until Day 28 (Figure 1A–C). Comparison between the muscles on Day 7 showed that the diaphragm expressed greater mRNA levels of IL-6 and MCP-1 than the TA and tongue, and greater levels of IL-1 β than the tongue (Figure 1A–C). These data suggest that the diaphragm tended to express greater mRNA levels of proinflammatory cytokines than the TA and tongue at an early stage of chronic inflammation (Figure 1A–C). Taken together, the findings mentioned earlier suggest that aspiration pneumonia induces the production of proinflammatory cytokines from the respiratory, skeletal, and swallowing muscles.

Effects of the aspiration challenge on the activation of calpain and caspase-3 in the respiratory, skeletal, and swallowing muscles.

Proinflammatory cytokines induce the activation of calpain and caspase-3, which cleave myofibrillar protein. We evaluated the activation of calpain and caspase-3 by determining the patterns of fodrin immunoreactivity on Western blot of whole muscle homogenates. The fodrin immunoreactive band of 120 kDa represents a specific cleavage product of caspase-3, while degradation of fodrin by calpain (and to a lesser extent caspase-3) results in bands of 145–150 kDa.¹² In the diaphragm, levels of 120 kDa and 145–150 kDa bands were greater on Days 7, 14, and 21 than in controls (Figure 2A, 2D, and 2E), suggesting greater activation of caspase-3 and calpains on these days. In the TA, the levels of bands were greater on Days 7 and 14 than in controls (Figure 2B, 2D,

Figure 1 Effects of aspiration challenge on the expression levels of proinflammatory cytokines in the muscles. (A–C) Quantitative real-time polymerase chain reaction was used to assess the expression levels of proinflammatory cytokines, IL-1 β (A), IL-6 (B), and MCP-1 (C) in the diaphragm, TA, and tongue. Cont, control group; 7d, 7 day challenged group; 14d, 14 day challenged group; 21d, 21 day challenged group; 28d, 28 day challenged group; TA, tibialis anterior; mRNA, messenger RNA; * $P < 0.05$ versus controls. # $P < 0.05$ versus the diaphragm treated for same days. $n = 6$ –8 in each group. Representative data of two independent experiments.

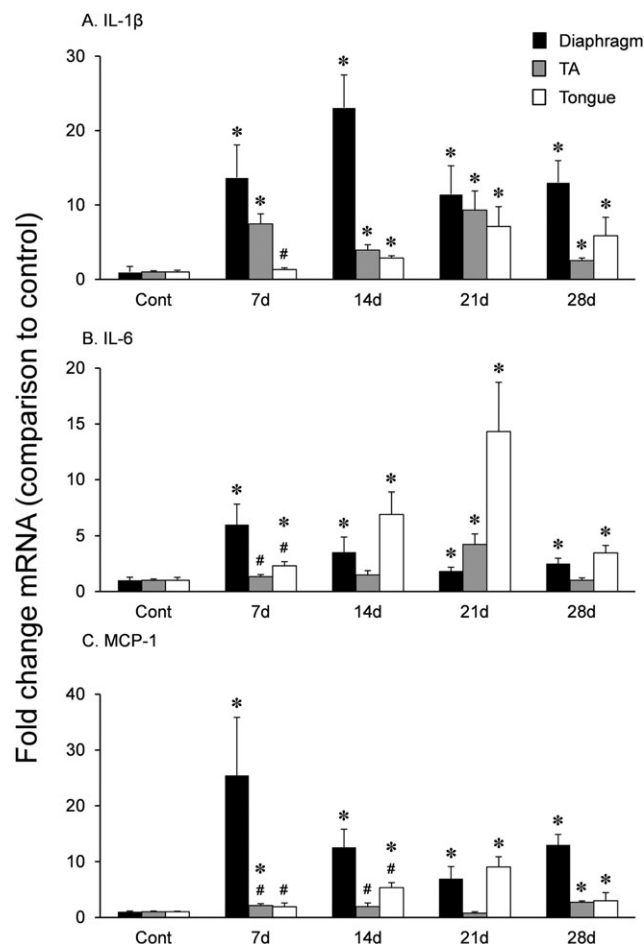
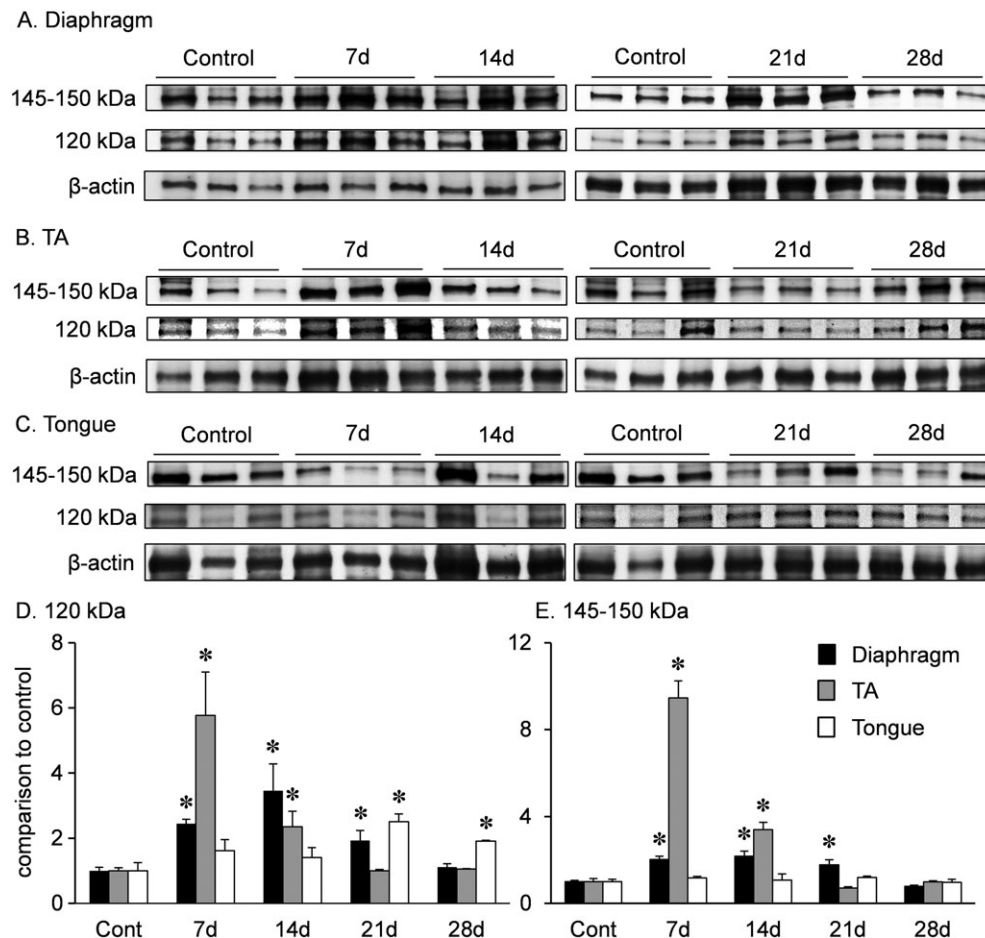


Figure 2 Effects of the aspiration challenge on protease activities in the muscles. Western blot images of fodrin immunoreactive bands at 120 kDa (cleavage product of caspase-3) and 145–150 kDa (predominately calpain-mediated cleavage product) in the diaphragm (A), TA (B), and tongue (C). Quantification of western blot images (expressed as fold change relative to the mean control value) of fodrin immunoreactive bands at 120 kDa (D) and 145–150 kDa (E) in the diaphragm, TA, and tongue. Cont, control group; 7d, 7 day challenged group; 14d, 14 day challenged group; 21d, 21 day challenged group; 28d, 28 day challenged group; TA, tibialis anterior; * $P < 0.05$ versus controls. $n = 3$ in each group. Representative images of three independent experiments.



and 2E). In the tongue, the levels of the 120 kDa bands were greater on Days 21 and 28 than in controls suggesting the activation of caspase-3 (Figure 2C and 2D). These results suggest the activation of calpain and caspase-3 in the diaphragm and TA, and the activation of caspase-3 in the tongue after the aspiration challenge.

Effects of the aspiration challenge on the activation of ubiquitin-proteasome system.

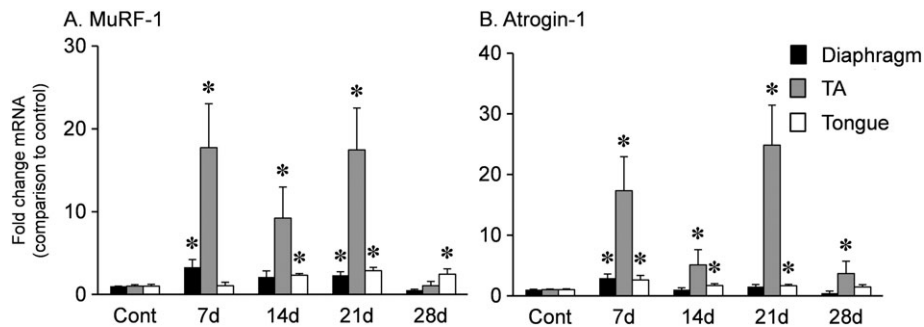
The activated ubiquitin-proteasome system degrades cleaved myofibrillar proteins. We evaluated the activation of the ubiquitin-proteasome system by the mRNA expression levels of two skeletal muscle E3 ubiquitin ligases, MuRF-1 and atrogin-1. In the diaphragm, the expression levels of MuRF-1 and atrogin-1 were greater than in controls on Day 7

(Figure 3A and 3B). The MuRF-1 levels also were greater on Day 21. In the TA, the expression levels of MuRF1 and atrogin-1 were greater on Days 7, 14, and 21 (Figure 3A and 3B). In the tongue, the expression levels of MuRF-1 were greater than in controls on Days 14, 21, and 28, whereas the levels of atrogin-1 were greater on Days 7, 14, and 21 (Figure 3A and 3B). These results suggest the activation of the ubiquitin-proteasome system after the aspiration challenge.

Effects of the aspiration challenge on the activation of autophagy.

To examine the involvement of autophagy, we evaluated the mRNA expression levels of prototypical genes involved in autophagy such as Bcl2/adenovirus E1B 19 kDa interacting

Figure 3 The aspiration challenge affects the expression levels of muscle-specific E3 ubiquitin ligases in muscles. Quantitative real-time polymerase chain reaction determined the expression levels of the muscle specific E3 ubiquitin ligases, MuRF-1 (A) and atrogin-1 (B) in the diaphragm, TA, and tongue. Cont, control group; 7d, 7 day challenged group; 14d, 14 day challenged group; 21d, 21 day challenged group; 28d, 28 day challenged group; MuRF-1, muscle RING-finger protein 1; mRNA, messenger RNA; TA, tibialis anterior; * $P < 0.05$ versus controls. $n = 6-8$ in each group. Representative data of two independent experiments.



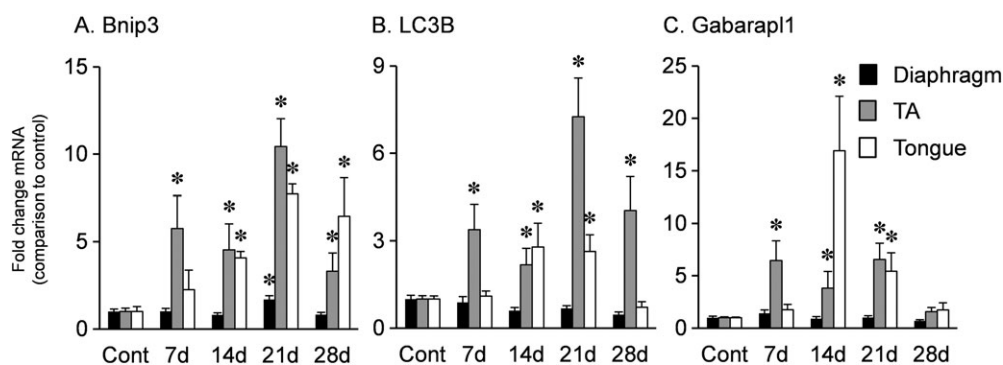
protein 3 (Bnip3), microtubule-associated protein 1 light chain 3B-II (LC3B-II), and GABA(A) receptor-associated protein like 1 (Gabap1). In the diaphragm, the aspiration challenge did not augment the expression levels at most of the time points (Figure 4A–C). An exceptional augmentation of the expression level of Bnip3 was detected on Day 21 (Figure 4A). In contrast, the expression levels of the three genes in the TA were greater at most of the time points than in controls (Figure 4A–C). In the tongue, the expression levels of the three genes were greater on Days 14 and 21 than in controls (Figure 4A–C). The expression level of Bnip3 was also greater on Day 28. In addition, we evaluated the activation of autophagy by detecting Bnip3 immunoreactivity on Western blot of whole muscle homogenates. Bnip3 immunoreactive bands are predicted to be detected around 22–28 kDa and their dimeric forms around 50–55 kDa. Western blot for the diaphragm showed greater levels of Bnip3 immunoreactive bands on Day 21 (Figure S1A, S1D, and S1E). Western blot for the TA showed greater

immunoreactive bands than controls on Days 7, 14, 21, and 28 (Figure S1B, S1D, and S1E), and for the tongue on Days 21 and 28 (Figure S1C, S1D, and S1E). Most of the above findings in western blots were consistent with the Q-RT-PCR data. These results suggest the involvement of autophagy in the TA and the tongue, and weak or little involvement in the diaphragm.

Effects of the aspiration challenge on muscle atrophy.

To determine the impact of above findings on the atrophy of muscle fibres, the frequency distribution of the cross-sectional area for individual myofibers was evaluated using haematoxylin and eosin sections of the muscles. In the diaphragm, myofibers showed a leftward shift to a greater proportion of smaller fibres in the 28 day challenged group

Figure 4 Effects of the aspiration challenge on activation of autophagy-related genes in the muscles. (A–C) Quantitative real-time polymerase chain reaction determined the expression levels of autophagy related genes, Bnip3 (A), LC3B (B), and Gabap1 (C) in the diaphragm, TA, and tongue. Cont, control group; 7d, 7 day challenged group; 14d, 14 day challenged group; 21d, 21 day challenged group; 28d, 28 day challenged group; Bnip3, Bcl2/adenovirus E1B 19 kDa interacting protein 3; LC3B, light chain 3B; Gabap1, GABA(A) receptor-associated protein like 1; mRNA, messenger RNA; TA, tibialis anterior; * $P < 0.05$ versus controls. $n = 6-8$ in each group. Representative data of two independent experiments.



(Figure 5A). Myofibers in the TA and the tongue showed similar trends (Figure 5B and 5C). These results suggest that aspiration challenge induced atrophy in the diaphragm, TA, and tongue.

Aspiration pneumonia induces muscle atrophy in human patients.

To analyse the effects of aspiration pneumonia on muscle atrophy in human patients, we evaluated the cross-sectional

area of a dorsal muscle group at the twelfth thoracic vertebra level using CT scan (Figure 6A). Table 2 shows general characteristics and comorbidities of the patients at admission. We routinely evaluate the pneumonia by CT scan at admission to (before the treatment) and at discharge from (after the treatment) the hospital. The cross-sectional areas before the treatment were set as 100%. The cross-sectional areas were reduced to 84.4% after treatment compared with before the treatment (Figure 6B). Mean duration between the CT scans was 20.6 days. These results suggest that aspiration pneumonia induces muscle atrophy in human patients.

Figure 5 The aspiration challenge induces muscle atrophy. (A–C) Representative images and frequency distributions of fibre size in the diaphragm (A), tibialis anterior (TA) (B), and tongue (C) isolated from controls (open bars) or 28 day challenged mice (solid bars). *n* = 6–8 in each group. Representative data of 2 independent experiments. Scale bar: 100 μ m.

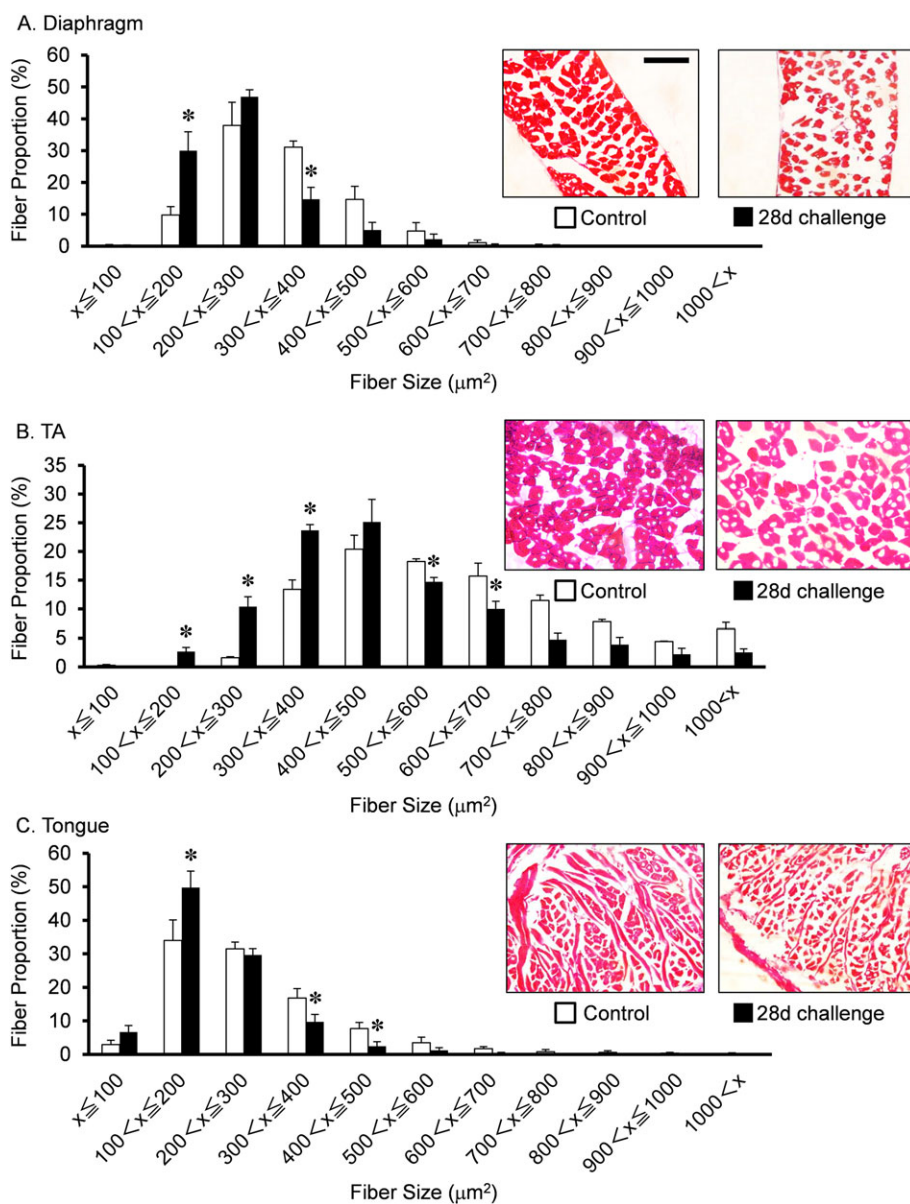


Figure 6 Aspiration pneumonia induces muscle atrophy in aged patients. (A) Cross sectional areas of the dorsal muscle group at Th 12 vertebral level (surrounded by red lines) were measured. (B) The cross sectional area in (A) before the treatment was set as 100% and relative change is shown after the treatment. * $P < 0.05$ versus before the treatment. $n = 8$.

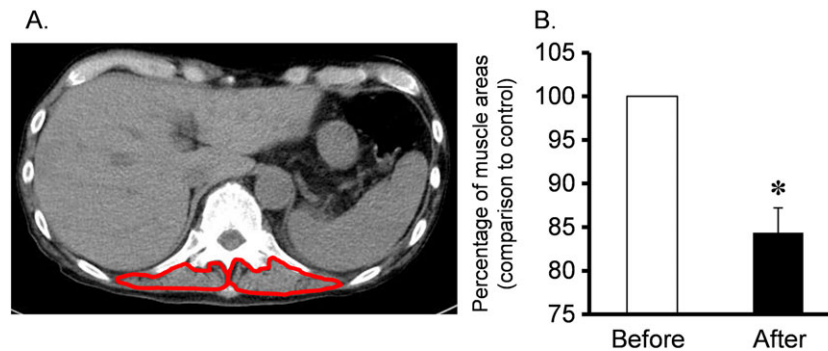


Table 2 General characteristics and comorbidities of study participants

Characteristics	Total sample ($n = 8$)
Age, mean \pm SE	79.3 \pm 2.2
Female, n (%)	1 (12.5)
Body mass index, mean \pm SE (kg/m^2)	20.1 \pm 2.3
Number of drugs, mean \pm SE	9.7 \pm 2.4
Hypertension, n (%)	5 (62.5)
Diabetes, n (%)	3 (37.5)
Chronic obstructive pulmonary disease, n (%)	2 (25)
Stroke, n (%)	4 (50)
Coronary heart disease, n (%)	3 (37.5)
Serum albumin at admission, mean \pm SE (g/dL)	2.8 \pm 0.16
Serum haemoglobin at admission, mean \pm SE (g/dL)	11.8 \pm 0.87
Serum CRP at admission, mean \pm SE (mg/dL)	16.0 \pm 4.6

CRP, C-reactive protein.

Discussion

In this study, aspiration challenge was followed by increased expression levels of proinflammatory cytokine genes in the muscles. Aspiration challenge activated muscle proteolysis pathways, which resulted in muscle atrophy in the mouse model. These data were confirmed in human patients with aspiration pneumonia by CT scans that showed muscle atrophy.

Aspiration pneumonia increased the expression levels of proinflammatory cytokines in the diaphragm and the TA. As far as we know, the cytokine-producing capacity of muscle fibres in the swallowing muscles such as the tongue is not known. In this study, we showed that aspiration pneumonia increased the expression levels of proinflammatory cytokines in the tongue, suggesting the tongue as one of the sources of myokines.

In majority of the previous studies, the duration of inflammation was mostly 1–4 days.^{12,14,20,25} In some studies,

the duration of inflammation was relatively long for 7–14 days.^{26,27} These studies evaluated the effects of acute or subacute inflammation on the diaphragmatic atrophy. In this study, the duration of inflammation in the mouse model was 28 days. This chronic inflammatory aggression may induce a host immune response and further inflammation. Indeed, in this model, our previous study showed that the bronchial lymph node weight and numbers of leukocytes in the bronchioalveolar lavage fluid peaked on Days 21 and 28.¹ These results may reflect induction of the host immune response. Expression levels of proinflammatory cytokines in the lung were greater than control for IL-1 β on Days 7 and 14, for IL-6 on Days 14, 21, and 28, and for MCP-1 on Days 14 and 21.¹ These expression levels of cytokines on Days 21 and 28 may reflect more advanced inflammation. Apart from most of the previous studies, this study evaluated the effects of chronic inflammation on atrophy of respiratory, skeletal, and swallowing muscles.

Previous studies described the possibility that the diaphragm is more predisposed to inflammatory responses than other muscles such as the TA or EDL.^{12,14} In a mouse sepsis model, endotoxin treatment *in vivo* induced greater levels of proinflammatory cytokines in the diaphragm than in the TA.¹⁴ In this study, the inflammation was chronic rather than acute. However, at an early stage such as Days 7 and 14, the expression levels of proinflammatory cytokines in the diaphragm were greater than in other muscles examined. This greater response to inflammatory stimulation may explain the vulnerability of the diaphragm in part.

During aspiration pneumonia, calpain and caspase-3 pathways were activated in the diaphragm and TA, and caspase-3 pathway was weakly activated in the tongue. The ubiquitin-proteasome system was activated in all three muscles. The autophagy pathway was activated in the TA and tongue, and very weakly in the diaphragm. Similarly, in the acute inflammation of mouse sepsis models, the activation of autophagy was weaker in the diaphragm than in the TA.^{20,25}

The underlying reasons for these differences are presently unclear. One possible factor that may explain the difference is exposure of the diaphragm to high stimulation frequency and intermittent stretch during breathing.²⁵ Mechanical stimulation to muscles induces the activation of mammalian target of rapamycin (mTOR) signalling.²⁸ Because mTOR has an inhibitory effect on autophagy, the activation of mTOR may influence the activation of autophagy in the diaphragm.²⁹ Another possible factor is the generation of reactive oxygen species (ROS). Mitochondrially derived ROS promote autophagy in muscles, and net mitochondrial ROS release is greater in the TA than in the diaphragm.²⁰ Overall, the findings mentioned earlier suggest differential mechanisms of atrophy between the muscles in response to aspiration challenge.

Because aspiration pneumonia is a disease of aged patients, we tried to establish an aspiration pneumonia mouse model using 1-year-old mice. Surprisingly, the aged mice were very susceptible to the aspiration challenge compared with 7- to 10-week-old mice. Respecting ethical guidelines, we ceased our trial to establish this model at the preliminary experimental stage.

Reduced mass and function of the swallowing muscles contribute to weakness in the swallowing function.³⁰ Reduced tongue pressure was described as a typical change of aged people.³⁰ Especially, the tongue strength was associated with the aspiration status.³¹ Thus, atrophy of the tongue by aspiration pneumonia might be one of the causes that induces repeated onsets of aspiration pneumonia. Moreover, among intensive care unit patients treated with mechanical ventilation, the presence of impaired swallowing function or dysphagia was related to death.^{30,32} These data suggest the importance of the tongue and other swallowing muscles in maintaining physical condition.

Aspiration pneumonia induced muscle atrophy in the preclinical model and in the human patients. These results suggest that aspiration pneumonia causes secondary sarcopenia. Cough requires the activation of respiratory muscles. Diaphragmatic atrophy by aspiration pneumonia may weaken the force of cough to expectorate sputum or mis-swallowed contents, which can be connected to the repetition of pneumonia. The tongue plays a central role in swallowing. The atrophy in the tongue may weaken the swallowing function. Indeed, sarcopenia is a risk factor for dysphagia in aged people.²² Taken together, the muscle atrophy induced by aspiration pneumonia may increase the risk of repeated onset of aspiration pneumonia in frail, aged people. Thus, repetitions of aspiration pneumonia may induce further muscle atrophy. A previous study presented a muscle hypothesis in which alterations of skeletal muscle structure and function lead to overactive ergoreflex, which exaggerates the hemodynamics, ventilatory effects, and symptomatic dyspnea.^{33,34} Similarly, we suggest that the systemic consequence of skeletal muscle loss in aspiration pneumonia is a vicious cycle of repeating onsets of aspiration pneumonia

and further muscle atrophy. To interrupt this vicious cycle, maintaining or increasing the muscle mass may have potential as a new strategy to treat aspiration pneumonia.

Under acute inflammation such as sepsis, the presence of muscular weakness including the dysfunction of respiratory muscles has been reported in animal models and patients.^{35–37} Under these conditions, the pathophysiology of muscle weakness such as proteolysis has been elucidated to a large extent using animal models.³⁵ Recent human studies have found a remarkable degree of similarity with the animal model data, suggesting the possibility of extrapolating the animal model data to the bedside.³⁸ In this study, we tried to evaluate similarities between the animal model and clinical data, which were confirmed in part. To further evaluate the similarities, we are now expanding human studies by investigating larger numbers of patients with aspiration pneumonia.

We showed presence of great levels of proinflammatory cytokines induced by aspiration challenge as a first step of muscle atrophy. However, muscle atrophy occurs under other various pathological conditions such as hypoxia, malnutrition, and inactivity, which also activate muscle proteolysis pathways.^{19,39} In mice, the aspiration challenge induced hypoxia and body weight loss in addition to inflammation.¹ Symptoms associated with pneumonia such as general fatigue, fever, and hypoxia may reduce food intake and physical activity. Therefore, potential mechanisms of body weight loss in this model may include malnutrition and immobility. Taken together, the mechanisms of muscle atrophy in this model may include hypoxia, and possibly malnutrition and immobility in addition to chronic inflammation. Medical nutrition for patients with chronic obstructive pulmonary disease and physical activity has been suggested to be clinically beneficial for muscle atrophy.^{40–43} Therefore, nutritional support and exercise training may have potential as therapeutic interventions for aspiration pneumonia to prevent and/or to improve muscle atrophy.

Because oral feeding of aspiration pneumonia patients is generally prohibited at admission, the progression of malnutrition may worsen the muscle atrophy. We compared the concentrations of serum albumin and haemoglobin as an indication of malnutrition. The lowest serum albumin concentration during the treatment, 2.5 ± 0.15 (g/dL, mean \pm SE), had a tendency to be lower than the concentration at admission, 2.8 ± 0.16 (g/dL, mean \pm SE), however, the difference was not significant. The concentration of haemoglobin showed similar results. Its lowest value was 10.1 ± 1.2 (g/dL, mean \pm SE) during the treatment and 11.3 ± 1.8 (g/dL, mean \pm SE) at admission. Because our objective was to evaluate the muscle mass, and comparison of the blood tests was not our primary objective, we enrolled eight patients in this study. We are now evaluating the presence of malnutrition in a greater number of patients.

Some of our study limitations should be considered. We could not include a positive control group for hospitalized

patients that show atrophy of skeletal muscles other than from aspiration pneumonia. A previous study reported the incidence of sarcopenia among hospitalized aged patients.⁴⁴ Because the methods to evaluate the muscles were completely different, we could not compare the degree of muscle atrophy with the previous study or other diseases. Gross observation of mice under aspiration challenge showed that mice became lean with decreased physical activity. These characteristics might be similar to the muscle atrophy induced by cancer and glucocorticoids.^{45–47} Reduced body weight by the aspiration challenge may reflect the lean appearance of mice.¹ For physical activity, facility limitations did not allow us to apply tracking devices to quantify the locomotor activity, as previously shown.⁴⁶ We evaluated the diaphragm as a respiratory muscle. However, we did not evaluate other respiratory muscles or accessory inspiratory/expiratory muscles.

In conclusion, we assessed the muscle atrophy in the respiratory, skeletal, and swallowing systems in a preclinical animal model and in human patients with aspiration pneumonia. The results suggest the relevance of this model in such patients. A better understanding of the role of muscles in the pathogenesis of aspiration pneumonia may provide a new target of analysis and therapies for aspiration pneumonia, which is a fatal and common disease in aged patients.

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Online supplementary material

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Effects of the aspiration challenge on the activation of autophagy-related protein in muscles. (A–C) Western blot images of autophagy related protein Bnip3 in the diaphragm (A), TA (B), and tongue (C). Bnip3 immunoreactive bands are predicted to be detected around 22–28 kDa and around 50–55 kDa, which 50–55 kDa bands are dimeric forms of 22–28 kDa bands. (D and E) Quantification of western blot images (expressed as fold change relative to the mean control value) of Bnip3 immunoreactive bands at 22–28 kDa (D) and 50–55 kDa (E) in the diaphragm, TA, and tongue. Cont, control group; 7d, 7-day challenged group; 14d, 14-day challenged group; 21d, 21-day challenged group; 28d, 28-day challenged group; * $P < 0.05$ versus controls. $n = 3$ in each group. Representative images of 3 independent experiments.

Conflict of interest

The authors declare no competing financial interests.

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