# Age-related changes in endoplasmic reticulum stress response-associated protein expression in rat tibial nerves

MASAHIRO SAKITA[1], WATARU ISOBE[1,2], KOJI NONAKA[3], SHINICHIRO MURAKAMI[4], RYO MIYACHI[5], KENTO SAKANE[1], SAKI SUGIMOTO[1], AIRI YAMAGUCHI[1] and KOKI YAMAMOTO[1]

[1]Department of Physical Therapy, Faculty of Health Sciences, Kyoto Tachibana University, Kyoto 607-8175, Japan; [2]Department of Rehabilitation, Mitsubishi Kyoto Hospital, Kyoto 615-8087, Japan; [3]Department of Rehabilitation, Faculty of Health Care Sciences, Naragakuen University, Nara 631-0003, Japan; [4]Department of Physical Therapy, Faculty of Health Care Sciences, Himeji-Dokkyo University, Himeji, Hyogo 670-0896, Japan; [5]Department of Physical Therapy, Faculty of Health Care Sciences, Hokuriku University, Kanazawa, Ishikawa 920-1154, Japan

Received October 17, 2024; Accepted January 13, 2025

DOI: 10.3892/br.2025.1928

**Abstract.** In age-related peripheral neurodegeneration, changes in the promotion or inhibition of endoplasmic reticulum (ER) stress response related to the ubiquitin-proteasome degradation system (UPS), autophagy and apoptosis signaling factors during aging remain unclear. In the present study, the expression of ER stress response signaling-related protein factors was examined in tibial nerves during aging in rats. Tibial nerves were extracted from continuously housed rats at 20, 50, 70, 90 and 105 weeks of age. Expression of factors associated with ER stress-related degradation, including X-box binding protein 1 (XBP1s), eukaryotic translation initiation factor 2 subunit 1 (eIF2α), Beclin-1 (Becn1), and Caspase-3 (Casp3); ER stress-related repair, including binding immunoglobulin protein [also known as 78 kDa glucose-regulated protein (BiP/GRP78)], protein disulfide isomerase (PDI), brain-derived neurotrophic factor (BDNF) and the inflammatory cytokine IL6, was assessed by western blotting of tibial nerves from rats in each age group. Expression of XBP1s and Becn1, which promote UPS and autophagy, decreased significantly after 50 weeks of age. However, expression of eIF2α and Casp3, which inhibit new protein biosynthesis and promote apoptosis, increased significantly after 50 weeks. Expression of BiP/GRP78 and PDI, which are refolding factors for denatured proteins, showed a significant decrease after 50 (or 70) weeks of age. The expression of BDNF, a ligand protein for the repair cascade, showed a significant increase after 70 weeks of age, while that of IL6 increased significantly after 50 weeks of age. These results indicate that ER stress-related degradation (UPS and autophagy) and refolding repair functions are reduced in rat tibial nerves after 50 weeks, followed by enhanced apoptosis and inflammation. These findings shed light on the progression of age-related peripheral neurodegeneration in rats.

*Correspondence to:* Professor Masahiro Sakita, Department of Physical Therapy, Faculty of Health Sciences, Kyoto Tachibana University, 34 Oyakeyamada, Yamashina, Kyoto 607-8175, Japan
E-mail: sakita@tachibana-u.ac.jp

*Abbreviations:* ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; Becn1, Beclin-1; BiP/GRP78, immunoglobulin heavy chain binding protein or glucose regulatory protein 78; Casp, caspase; DRG, dorsal root ganglia; EDL, extensor digitorum longus; ER, endoplasmic reticulum; eIF2α, eukaryotic initiation factor as novel protein synthesis inhibitor factor-2α; FAM134B, family with sequence similarity 134, member B; IL6, interleukin-6; JNK1, c-Jun N-terminal kinase; MF, myelinated fiber; OD, optical density; PDI, protein disulfide isomerase; PVDF, polyvinylidene fluoride; Sol, soleus; TNF-α, tumor necrosis factor-α; UPS, ubiquitin-proteasome degradation system; XBP1s, X-box binding protein 1

*Key words:* endoplasmic reticulum, tibial nerve, neurodegeneration, aging, ubiquitin-proteasome degradation system, apoptosis, autophagy

## Introduction

Sarcopenia, or the age-related loss of muscle mass and strength, increases the risk of functional decline (1,2) and dementia (3). As it results in poor balance, sarcopenia is a major risk factor for falls in community-dwelling older adults (4-6). Such falls in the elderly can cause femoral neck and other fractures, subsequently reducing their ability to carry out activities with daily living (7,8). Age-related sarcopenia is accompanied by a regression of myelinated fibers (MFs) (9,10). Furthermore, electrophysiological experiments show that a decline in peripheral nerve function precedes the decline in muscle function during aging (11). These findings suggest that impaired balance and an increased risk of falls may be due to peripheral nerve degeneration, in addition to sarcopenia.

Histological studies of lumbar and cervical dorsal root ganglia (DRG), obtained from 12 human autopsies (age range 60-70 years) of individuals without associated diseases of the central or peripheral nervous system, have reported a 35% reduction in the number of neurons relative to those

obtained from autopsies of individuals aged <10 years (12). Additionally, the number of fibers in the peroneal nerve with diameters >8 $\mu$m decreased with the age of autopsy subjects aged 17-72 years (13). In a study of DRG in the cervical and lumbar spinal cord of 12-week and 120-week-old rats, the latter exhibited a 12% decrease in the total number of neurons and a 16% decrease in the percentage of MFs in cross-sectional scores, indicating age-related loss of MFs and atrophy (14). Therefore, it appears that selective atrophy and loss of MFs in peripheral nerves are associated with aging. In a recent study, the tibial nerves of 20-, 70-, and 97-week-old rats, which had been continuously kept in the same environment, were examined histologically. MF axon diameter and myelin sheath thickness in the 97-week-old physically inactive rats were significantly reduced relative to that in the 20- and 70-week-old rats. However, the 70-week-old rats showed only a mild decrease in MF axon diameter and myelin sheath thickness relative to 20-week-old rats, with no other pathological deformities (15). Furthermore, in a previous cross-sectional study, which examined the morphology of the tibial nerve of 24- to 108-week-old mice (physical activity or lack thereof was unclear), MF morphology remained stable until 48 weeks, with only slight changes between 48 and 80 weeks. Moreover, the MF morphology showed significant fiber loss decreased size, and increased irregular shape from 80 weeks to 108 weeks (9,16). Based on these histological and morphological findings in rodents, a marked progression of atrophy and degeneration of axons and myelin sheaths is predicted between ~70 and 80 weeks of age.

In an electrophysiological study of age-related changes in human peripheral nerves, action potential amplitudes of sensory nerves were reduced only in the peroneal (-73%) and median (-38%) nerves in the older adult group (63-80 years) relative to the young adult group (21-29 years). By contrast, there was a marked reduction in conduction velocity and action potential amplitude in both motor and sensory nerves in the group of individuals older than 80 years (17). In several aging studies involving mice, increased myelin sheath thickness, number of MFs with large diameters, and sensory nerve conduction velocities were observed in mice up to the age of 48 weeks. This was accompanied by slight reductions in these parameters in mice aged 48-80 weeks and marked decrements in mice >80 weeks (9,16,18). Thus, age-related dysfunction of MFs in peripheral nerves is associated with histological degeneration and induces a delayed onset of lower limb muscle activity and reduced action potentials during postural recovery in response to postural disturbance. Moreover, biochemical signaling of age-related degeneration in peripheral nerves is hypothesized to precede the histological and physiological changes in peripheral nerves associated with aging.

The ubiquitin-proteasome system (UPS) (19) or autophagy (20,21) is a major proteolytic mechanism. Defects in these mechanisms are known to be involved in neurodegeneration. When injured or unfolded proteins in abnormal conformations accumulate in the endoplasmic reticulum (ER), the UPS induces either protein repair, degradation, or apoptosis as a stress avoidance mechanism known as the ER stress response (22). Within the ER, damaged and unfolded proteins undergo ubiquitination and are subsequently returned from the ER to the cytoplasm, where they are transported to proteasomes for degradation (23). Autophagy also degrades misfolded protein aggregates in defective cellular organelles and lysosomes, a process that is part of the ER stress response (20,21). Under sustained or excessive ER stress that cannot be regulated by UPS or autophagy, cells eventually undergo apoptosis (24). Thus, UPS, autophagy and apoptosis, which are all processes of the ER stress response, are important for processing defective proteins and maintaining protein homeostasis. Studies in *Drosophila* show that the expression of UPS and autophagy-related factors in the proteolytic system declines, and ubiquitinated proteins (defective proteins) accumulate in the brain with age (25). In rodent and human cells, proteolytic function of lysosomes declines with age and subsequent impairment of autophagy flow exacerbates cellular damage leading to the development of age-related diseases (26-28). However, the age at which the decline in UPS and autophagy is initiated is not clear.

Increased age-related neuronal damage, unfolded proteins and apoptosis are associated with oxidative stress and free radical accumulation (29). Proteasome activity, which protects proteins, DNA and lipids from oxidative stress in spinal cord neurons, is markedly reduced in rats after middle age (30). Additionally, structural defects in axons, myelin and cell bodies of motoneurons in 80-week-old model mice were associated with increased oxidative stress and defects observed with normal aging in 120-week-old wild-type mice (31). Taken together, these findings suggest that age-related and ER stress-related inhibition of UPS/autophagy, promotion of apoptosis and increased oxidative stress promote neurodegeneration. However, the biochemical mechanisms underlying the regulation of UPS, autophagy and apoptosis during aging remain unclear.

The present study aimed to determine how the promotion or inhibition of ER stress-related UPS, autophagy and apoptotic pathways involved in neurodegeneration is altered during aging. Specifically, the expression levels of signaling-related factors of the ER stress response were analyzed in the tibial nerve of rats aged 20, 50, 70, 90 and 105 weeks.

## Materials and methods

*Animals*. A total of 44 male Wistar rats (mean weight, 330.2±9.7 g) (Japan SLC, Shizuoka, Japan) acquired at 10 weeks of age were housed continuously until they were 105 weeks old. They were randomly enrolled into the study at the following age groups: 20 weeks (n=11), 50 weeks (n=12), 70 weeks (n=12), 90 weeks (n=11) and 105 weeks (n=8). The rats were housed in plastic cages (two individuals per cage) and kept at a temperature of 22±2˚C with humidity of 40-60%. A 12/12-h light-dark cycle was maintained with lights on at 8:00 a.m. and off at 8:00 p.m., and they had access to food and water *ad libitum*. The present study was approved by the Animal Care and Use Committee of Kyoto Tachibana University (approval no. 19-10; Kyoto, Japan) and was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (National Research Council, 1996).

*Collection and preservation of nerve and skeletal muscle samples*. Randomly selected rats at each week of age were selected from the five age groups and anesthetized by

isoflurane inhalation (2.0%), followed by intraperitoneal injection of sodium pentobarbital (15 mg/kg) and heparin (10,000 IU/l) (15). The soleus (Sol) and extensor digitorum longus (EDL) muscles of the right hindlimb were extracted. Subsequently, saline was refluxed caudally at 37˚C from the abdominal aorta, and the nerve segment between the sciatic L4-L5 isthmus of the right hindlimb and the distal tibial nerve branching to the medial and lateral plantar nerves was removed (15). The rats were immediately euthanized via intraperitoneal administration of 120 mg/kg of sodium pentobarbital, and nerve samples were immediately immersed or frozen in isopentane cooled with liquid nitrogen for ~10 sec. From the aforementioned, Sol, EDL and tibial nerve samples were taken as part of the terminal procedure and animals were euthanized after sample collection. The samples were stored at -80˚C until further use.

*Tibial nerve sample preparation and assay for total protein volume*. A portion of the tibial nerve from the distal end of a frozen peripheral nerve sample of the right hindlimb was cut. It weighed 10-12 mg. One cut sample was lysed in 500 $\mu$l lysis buffer (cat. no. sc-24948A; RIPA Lysis Buffer System; Santa Cruz Biotechnology, Inc.) supplemented with 1 mM EDTA and 1 ml protease inhibitor cocktail and homogenized by sonication under ice-cold conditions. Lysed samples were centrifuged at 15,000 x g for 15 min at 4˚C and the total protein content in the supernatant was quantitated using the Bicinchoninic Acid Protein Assay (cat. no. TQ300A; Takara Bio, Inc.). The absorbance of each sample was observed at 560 nm using a microplate reader (Multiskan JX; Thermo Fisher Scientific, Inc.) to estimate total protein concentration. Each sample was diluted 2-fold to prepare the sample solution for electrophoresis and was denatured in sample buffer (0.5 M Tris HCl (pH 6.8), 20% glycerol, 1% β-mercaptoethanol, 1% SDS and 0.02% bromophenol blue) by boiling at 95˚C for 5 min and stored at -20˚C until subsequent assays.

*Western blotting*. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. For electrophoresis, 20 $\mu$l of sample solution (protein 1.60 $\mu$g/$\mu$l) was loaded into each well and electrophoresis was performed at 200 V for 80 min. The separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a wet blotter (cat. no. 1704070JA; Criterion Blotter with Plate Electrodes; Bio-Rad Laboratories, Inc.) at 50 V for 80 min. After blotting, the PVDF membranes were washed with TBST (25 mM Tris HCl, 0.15 M NaCl, 0.1% Tween-20, pH 7.4) for 3 min; this was followed by washing with deionized water for 3 min with gentle shaking. PVDF membranes were incubated with Ponceau S (cat. no. SP-4030; Apro Science Group; https://apro-s.com/) by gentle shaking for 5 min, followed by washing with deionized water for 2 min to ensure clear protein staining. Stained PVDF membranes were scanned, and images were captured using a personal computer. To decolorize protein staining, the PVDF membranes were incubated with 0.1 M NaOH by gentle shaking for 30 sec, followed by washing with TBST for 3 min. The PVDF membranes were incubated with a blocking solution (cat. no. T7132A; Western BLoT Blocking Buffer; Takara Bio, Inc.) thereafter for 1 h at room temperature. Subsequently, the membranes were washed gently with TBST for 5 min. Following this, the membranes were probed overnight (at 4˚C) with primary antibodies prepared in blocking solution.

In the present study, focus was addressed on the age-dependent changes in the expression of proteins related to the ER stress response in the tibial nerves. In a previous longitudinal study, histological and morphological analysis of the tibial nerves at 20, 70 and 97 weeks of age revealed only slight atrophy and degeneration of axons and myelin sheaths at up to 70 weeks, with a marked progression of degeneration after 70 weeks (15). Based on this finding, it was hypothesized in the present study that age-related changes in the expression of ER stress response-related proteins may be associated with these histological and morphological changes. However, after 70 weeks, these repair mechanisms are expected to decline, leading to the accumulation of damaged proteins and subsequent apoptosis. Specifically, protein repair mechanisms (for example the ubiquitin-proteasome system) and the ER stress response appear to be active and suppress these degenerative changes from 20 to 70 weeks of age. However, the function of these repair mechanisms declines above 70 weeks of age, after which denatured proteins accumulate and apoptosis is predicted to eventually progress. Therefore, the present study focused on changes in the expression of the following proteins associated with the ER stress response.

Primary antibodies against the following were used: anti-ER stress-related UPS and autophagy factors, apoptotic factors, new protein synthesis inhibitors, repair factors and inflammatory cytokines. The following proteins were employed as biomarkers: splicing X-box binding protein 1 (XBP1s) for UPS; Beclin-1 (Becn1) for autophagy; caspase-3 (Casp3) for apoptosis; eukaryotic initiation factor as novel protein synthesis inhibitor factor-2α (eIF2α); immunoglobulin heavy chain binding protein or glucose regulatory protein 78 (BiP/GRP78), protein disulfide isomerase (PDI) and brain-derived neurotrophic factor (BDNF) for repair; and interleukin-6 (IL6) as the inflammatory cytokine.

The following primary antibodies were used: XBP1s rabbit monoclonal antibody (1:1,000; cat. no. 82914; Cell Signaling Technology, Inc.), eIF2α rabbit monoclonal antibody (1:1,000; cat. no. 9079; biotinylated; Cell Signaling Technology, Inc.), Becn1 rabbit polyclonal antibody (1:1,000; cat. no. 11306-1-AP; Proteintech Group, Inc.), Casp3 rabbit polyclonal antibody (1:500; cat. no. GTX110543; GeneTex, Inc.), BiP/GRP78 rabbit polyclonal antibody (1:1,000; cat. no.11587-1-AP; Proteintech Group, Inc.), PDI rabbit polyclonal antibody (1:1,000; cat. no. 2446; Cell Signaling Technology, Inc.), BDNF mouse monoclonal antibody (1:1,000; cat. no. ab203573; Abcam) and IL6 rabbit monoclonal antibody (1:1,000; cat. no. ab259341; Abcam). After probing with the primary antibodies, PVDF membranes were washed three times (10 min/time) with TBST. They were subsequently incubated for 1 h at room temperature with the following secondary antibodies: goat anti-rabbit IgG HRP-conjugated antibody (cat. no. SA 00001-2; Proteintech Group, Inc.) at a dilution of 1:8,000 in blocking buffer for XBP1s, Casp3, PDI and IL6; and at 1:10,000 for Becn1; streptavidin HRP-conjugated antibody (cat. no. 3999; Cell Signaling Technology, Inc.) at a dilution of 1:10,000 in blocking buffer for eIF2α; and goat anti-mouse IgG HRP-conjugated antibody (cat. no. SA00001-1; Proteintech Group, Inc.) at a dilution of 1:8,000 in blocking buffer for BDNF. Then, the PVDF

membranes were washed three times (10 min/time) with TBST. Following this, the membranes were sealed within a plastic wrap containing chemiluminescence detection reagent (cat. no. RPN2232; Amersham ECL Prime; Cytiva) and allowed to react for 30 sec for the detection of target proteins. The chemiluminescence images of each target protein were acquired using a chemiluminescence imaging system (LumiCube; Liponics; http://www.lipopartners.jp/index.html) and captured on a personal computer using EOS Utility software (version 2.14; Canon Inc.) with an exposure time of 20 sec. Images captured on the personal computer were converted from 16 to 8 bit using ImageJ software (version 1.53; National Institutes of Health) and inverted. Luminescent bands corresponding to target proteins were marked with rectangles, and their chemiluminescence signal intensities were calculated as the optical density (OD). Additionally, the OD values of the target proteins were calculated as relative values by dividing them by the OD value for all the proteins observed in the Ponceau S staining image (32,33).

*Statistical analysis*. Physical characteristics (body weight, Sol and EDL weights) data for the five groups of rats are indicated as the mean ± standard error. Relative amounts of each target protein in each group were determined by setting the median value of the 20-week-old group as 1. Relative amounts of target proteins are shown as medians and interquartile ranges; comparisons of physical characteristics of the five groups of rats were made using one-way analysis of variance (ANOVA). Comparisons of the relative amounts of each target protein in the five groups were made using the Kruskal-Wallis test. Multiple comparisons using Tukey-Kramer post hoc test and Steel-Dwass test were performed when significant effects were identified by one-way ANOVA and Kruskal-Wallis tests, respectively. All statistical analyses were performed using R version 4.0.0 (R Foundation for Statistical Computing). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Physical characteristics*. The physical characteristics of the five experimental groups of rats in the present study are compared in Table I. ANOVA showed that body mass was affected by age ($P<0.01$). Subsequent results of multiple comparisons showed that the body masses of the four older groups of rats were significantly higher than those of the 20-week-old rats ($P<0.01$ for all four groups). Sol muscle masses of the five groups indicated no differences by age. The EDL muscle mass of the five groups was affected by age ($P<0.01$). Both the 50- and 70-week-old groups of rats had significantly greater mass than those in the 20-, 90- and 105-week-old groups ($P<0.01$).

*Comparison of the relative amounts of ER stress-related degradation factors by age*. Relative expression of XBP1s differed significantly among the five age groups of the rats ($\chi^2=24.4$, df=4, $P<0.01$) (Fig. 1A). XBP1s expression in the 105-week-old group (0.28, 0.15-0.31) was significantly lower than that in the 20- (1, 0.88-1.64) ($P<0.01$), 50- (2.5, 1.06-3.33) ($P<0.01$), 70- (1.22, 0.95-1.42) ($P<0.01$) and 90-week-old groups (1.1, 0.78-1.54) ($P<0.01$) (Fig. 1A).

Table I. Physical characteristics of rats aged 20, 50, 70, 90, and 105 weeks.

| | Groups | | | | | | |
|---|---|---|---|---|---|---|---|
| Parameters | 20-week-old (n=11) | 50-week-old (n=12) | 70-week-old (n=12) | 90-week-old (n=11) | 105-week-old (n=8) | F | P-value in ANOVA |
| Body mass (g) | 456.6±7.1 | 561.5±8.6[a] | 602.9±12.2[a] | 563.0±32.8[a] | 599.6±39.0[a] | F (4, 49) 7.76 | P<0.01 |
| Sol muscle mass (mg) | 168.7±6.6 | 190.3±8.7 | 183.9±10.2 | 180.3±8.8 | 180.6±7.3 | F (4, 49) 0.86 | Not significant |
| EDL muscle mass (mg) | 210.5±6.2 | 244.8±5.7[a] | 248.0±5.8[a] | 205.1±7.2[b,c] | 198.8±10.3[b,c] | F (4, 49) 11.5 | P<0.01 |

One-way analysis of variance (ANOVA) for body weight showed that it was affected by age. Multiple comparisons also showed that the body weights of rats in the four groups from the 50-week-old group onwards were significantly higher than that of those in the 20-week-old group. One-way ANOVA for Sol muscle mass showed no differences between age groups. Multiple comparisons showed a significant increase in the EDL mass of rats in the 50- and 70-week-old rats compared with those in the 20-week-old group. In addition, the EDL mass of rats in the 90- and 105-week-old groups was significantly lower than those in the 50- and 70-week-old groups. Results are presented as the mean ± standard error (SE). F values are provided along with degrees of freedom in parenthesis. [a]P<0.01 compared with 20-week-old group according to Tukey-Kramer post hoc test; [b]P<0.01 compared with 50-week-old group according to Tukey Kramer post hoc test; [c]P<0.01 compared with 70-week-old group according to Tukey Kramer post hoc test. Sol, soleus, EDL, extensor digitorum longus.

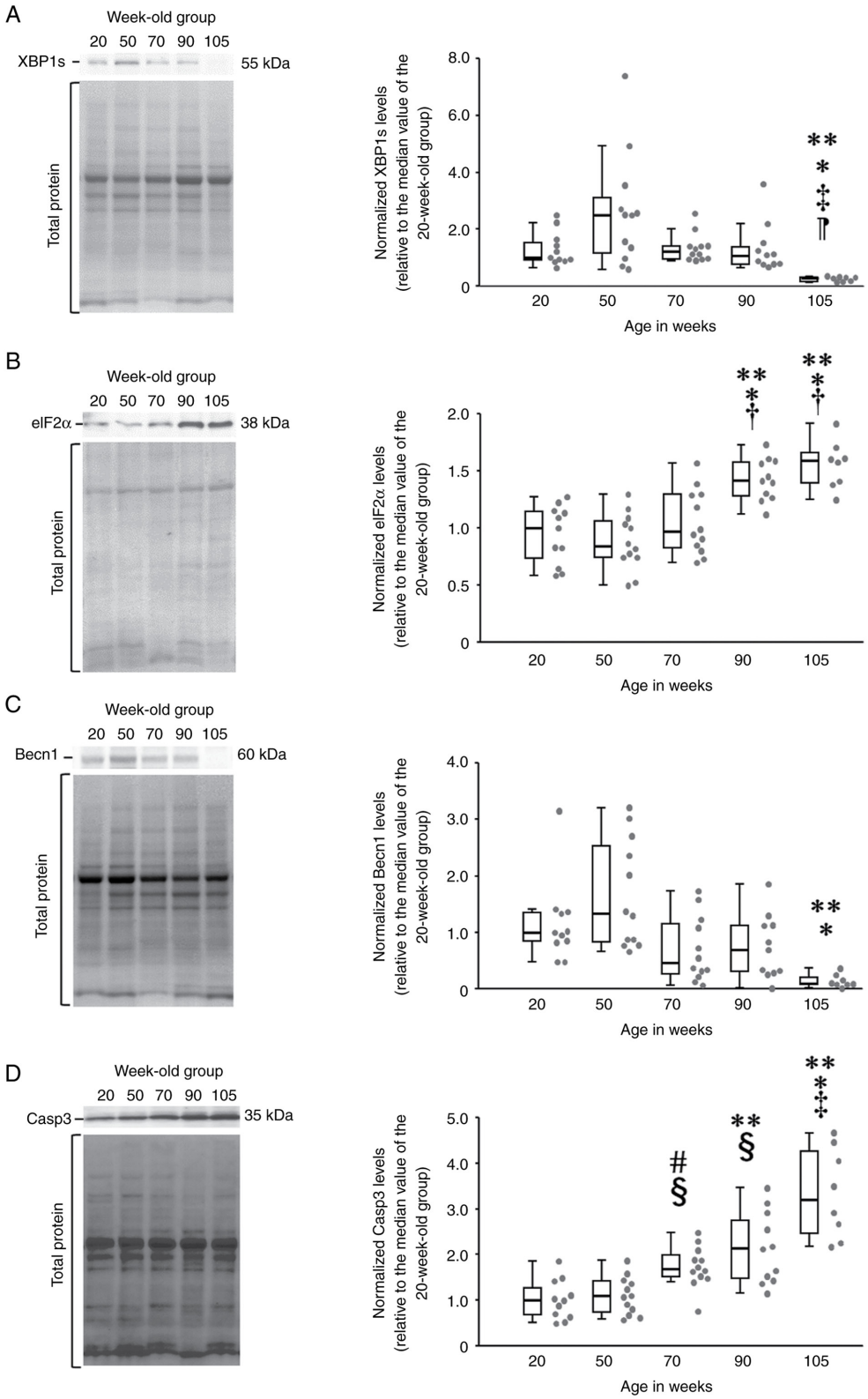


Figure 1. Comparison of relative concentrations of ER stress-related degradation proteins in aging rats. (A) In multiple comparisons (Steel-Dwass test), relative expression of XBP1s was significantly lower in rats in the 70-week-old and older groups (boxplot diagram, right). In the chemiluminescence image of XBP1s (left), the signal band of 50-week-old rats was the darkest, while those for rats older than 70 weeks were thinner. (B) Relative eIF2α expression in the Steel-Dwass test: rats in the 90- and 105-week-old groups showed significantly greater expression relative to those in other groups (boxplot diagram, right). Chemiluminescence images of eIF2α (left) showed that the signal bands of 90- and 105-week-old rats were significantly darker than those of the other three groups. (C) Relative Becn1 expression in the Steel-Dwass test: rats in the 105-week-old group showed significantly lower expression relative to those in other groups (boxplot diagram, right). The signal band of the 105-week-old rats was thinner than those of the other 4 groups, indicating lower relative expression. (D) Relative Casp3 expression in the Steel-Dwass test: relative Casp3 expression was significantly higher in rats in the 70-week-old and older groups (boxplot diagram, right). In the chemiluminescence image for Casp3 (left), the signal band gradually darkened with increasing age. The gray dots on the right side of the box-and-whisker plots indicate the dot plot for each group. $^{**}$P<0.01 vs. 20-week-old group; $^{*}$P<0.01 vs. 50-week-old group; $^{‡}$P<0.01 vs. 70-week-old group; $^{¶}$P<0.01 vs. 90-week-old group; $^{\#}$P<0.05 vs. 20-week-old group; $^{§}$P<0.05 vs. 50-week-old group; and $^{†}$P<0.05 vs. 70-week-old group. ER, endoplasmic reticulum; XBP1s, X-box binding protein 1; eIF2α, eukaryotic initiation factor as novel protein synthesis inhibitor factor-2α; Becn1, Beclin-1; Casp3, caspase.

Relative expression of eIF2α differed significantly among the five age groups of the rats ($\chi^2$=30.3, df=4, P<0.01) (Fig. 1B). eIF2α expression in the 105-week-old group (1.59, 1.39-1.68) was significantly higher than that in the 20- (1, 0.64-1.15) (P<0.01), 50- (0.84, 0.74-1.08) (P<0.01) and 70-week-old groups (0.97, 0.81-1.29) (P<0.05) (Fig. 1B). eIF2α expression in the 90-week-old group (1.43, 1.27-1.59) was significantly higher than that in the 20- (P<0.01), 50- (P<0.01) and 70-week-old (P<0.05) groups (Fig. 1B).

Relative expression of Becn1 differed significantly among the five age groups of the rats ($\chi^2$=23.6, df=4, P<0.01) (Fig. 1C). Becn1 expression in the 105-week-old group (0.1, 0.08-0.23) was significantly lower than that in the 20- (1, 0.83-1.37) (P<0.01) and 50-week-old groups (1.34, 0.80-2.62) (P<0.01) (Fig. 1C). Relative expression of Becn1 in the 70- (0.46, 0.25-1.2) (P<0.1) and 90-week-old (0.7, 0.29-1.13) (P<0.1) groups also tended to be lower than that in the 50-week-old group and higher than that in the 105-week-old group.

Relative expression of Casp3 differed significantly among the five age groups of the rats ($\chi^2$=32.8, df=4, P<0.01) (Fig. 1D). Casp3 expression in the 105-week-old group (3.21, 2.36-4.37) was significantly higher than that in the 20- (1, 0.64-1.43) (P<0.01), 50- (1.09, 0.71-1.43) (P<0.01), and 70-week-old groups (1.69, 1.49-2.05) (P<0.01) (Fig. 1D). In addition, Casp3 expression in the 90-week-old group (2.13, 1.44-2.93) was significantly higher than that in the 20- (P<0.01) and 50-week-old groups (P<0.01) (Fig. 1D). Furthermore, Casp3 expression in the 70-week-old group was significantly higher than that in the 20- (P<0.05) and 50-week-old groups (P<0.05) (Fig. 1D).

*Comparison of relative amounts of ER stress-related repair factors and inflammatory cytokines by age*. Relative expression of BiP/GRP78 differed significantly among the five age groups of the rats ($\chi^2$=18.9, df=4, P<0.01) (Fig. 2A). BiP/GRP78 expression in the 105-week-old group (0.65, 0.2-1.02) was significantly lower than that in the 50- (1.2, 1.03-1.33) (P<0.05) and 70-week-old groups (1.11, 0.98-1.28) (P<0.05) (Fig. 2A). In addition, BiP/GRP78 expression in the 90-week-old group (0.88, 0.82-1.02) was also significantly lower than those in the 50-week-old group (P<0.01) (Fig. 2A).

Relative expression of PDI differed significantly among the five age groups of the rats ($\chi^2$=22.6, df=4, P<0.01) (Fig. 2B). PDI expression in the 90-week-old group (0.43, 0.28-0.84) was significantly lower than that in the 50-week-old group (1.18, 0.85-2.05) (P<0.05) (Fig. 2B). PDI expression in the 105-week-old group (0.19, 0.14-0.40) was also significantly lower than that in the 20- (1, 0.68-1.79) (P<0.05), 50- (P<0.01) and 70-week-old groups (0.86, 0.50-1.22) (P<0.05) (Fig. 2B).

Relative expression of BDNF differed significantly among the five age groups of the rats ($\chi^2$=33.7, df=4, P<0.01) (Fig. 2C). BDNF expression in the 105-week-old group (14.8, 9.46-18.03) was significantly higher than that in the 20- (1, 0.32-1.85) (P<0.01) and 50-week-old groups (1.61, 0.36-3.46) (P<0.01) (Fig. 2C). BDNF expression in the 90-week-old group (9.17, 6.81-10.83) was also significantly higher than that in the 20- (P<0.01) and 50-week-old groups (P<0.01) (Fig. 2C).

Relative expression of IL6 differed significantly among the five age groups of the rats ($\chi^2$=21.5, df=4, P<0.01) (Fig. 2D). IL6 expression in the 105-week-old group rats (1.69, 1.47-1.91) was significantly higher than that in the 20- (1, 0.8-1.28) (P<0.05) and 50-week-old groups (1.34, 1.14-1.46) (P<0.05) (Fig. 2D). In addition, IL6 expression in the 90-week-old group (1.7, 1.43-1.76) was significantly higher than that in the 20- (P<0.01) and 50-week-old groups (P<0.05) (Fig. 2D). Furthermore, IL6 expression in the 70-week-old group (1.6, 1.28-1.91) was significantly higher than that in the 20-week-old group (P<0.05) (Fig. 2D).

## Discussion

In the present study, changes in the expression levels of degradation factors (associated with UPS, autophagy and apoptosis), repair factors, and the inflammatory cytokine IL6 induced by the ER stress response in aging rats, were examined to detect signs of age-related neurodegeneration. Among the physical characteristics, body mass of the rats in the four groups aged 50 weeks and older increased more than that of the 20-week-old rats. A previous study examining the physical parameters of aging male Wistar rats (n=402) housed in a controlled environment reported a rapid increase in body mass until the 69th week (mean body mass 629 g), with a gradual increase or maintenance until the 134th week (mean body mass 660 g), and a decrease thereafter (34). Similar to this finding, rats in the present study showed a rapid increase in mean body mass up to the age of 70 weeks, which was maintained thereafter. However, the body mass after 70 weeks (mean 588 g from 70 to 105 weeks of age) was ~70 g less than the mean body mass in the previous study (660 g). The body mass of rats is affected by several factors, such as handling, type and quality of food, density of animals in the cage, temperature, humidity, light regime and opportunities for active movement (34). Therefore, the difference between the body masses of the rats in the study by Nistiar *et al* (34) and the present study may have been influenced by several factors during the rearing process.

Comparisons of the Sol muscle masses for the five groups showed no differences with age. However, the 20-, 90- and 105-week-old rats had noticeably lower EDL masses than the 50- and 70-week-old rats. In experiments examining the pathophysiological characteristics of sarcopenia in young, adult, middle-aged and aged rats, typical signs such as shortening of single contractions, reduced relaxation rate, and impaired mobilization of motor units at stimulus frequencies above 50-60 Hz, were especially observed in the fast twitch muscle-dominant plantaris muscles of aged rats (35). Furthermore, mild signs of sarcopenia have been observed in predominantly slow twitch Sol muscles (35). In addition, sarcopenic muscles have altered myofibers, with a reduced type II/ I fiber ratio and decreased muscle mass and strength with age (36,37). These findings indicate that type II fibers are more susceptible to atrophy in muscles, and a predominantly fast-twitch component and is more likely to occur specifically in sarcopenia. In the present study, the Sol muscle, a slow-twitch dominant configuration, showed no loss in mass after 50 weeks of age, while the EDL, a fast-twitch dominant configuration, showed a marked loss in mass in age groups beyond 70 weeks, that is, at 90 and 105 weeks. The EDL mass of rats in the 90-week-old group was significantly less than that of rats in the 70-week-old group, suggesting that EDL mass decreased gradually and progressively after 70 weeks. In support of this

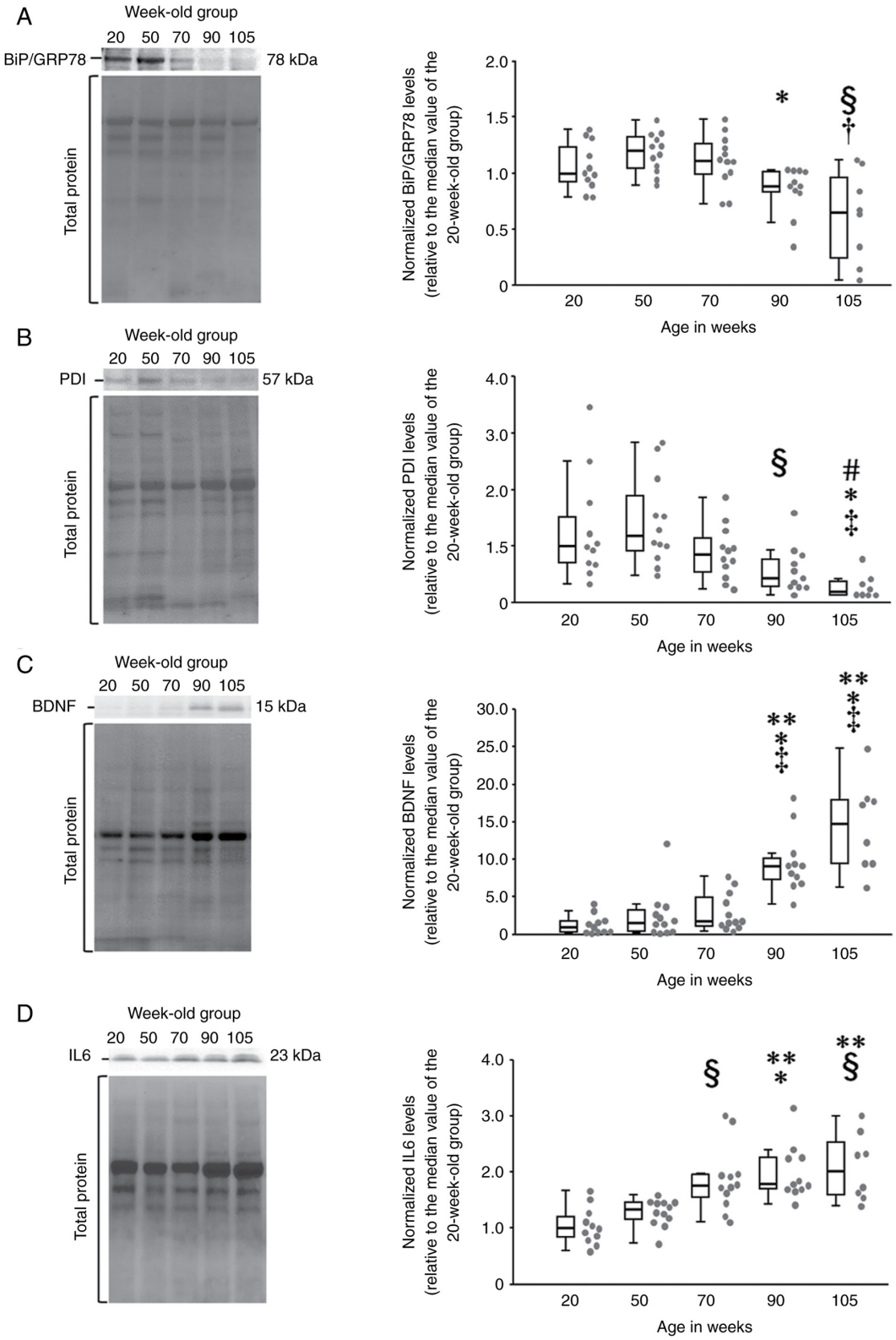


Figure 2. Comparison of the relative expression of ER stress-related repair proteins and the inflammatory cytokine IL6 in aging rats. (A) Multiple comparisons of the relative expression of BiP/GRP78 using the Steel-Dwass test showed significant reductions in rats in the 90- and 105-week-old groups (box plot diagram, right). In addition, the chemiluminescence image of BiP/GRP78 (left) shows that the signal bands of the 90- and 105-week-old rats are thinner than those of rats of other ages. (B) Relative PDI expression in the Steel-Dwass test: rats in the 90- and 105-week-old groups showed significantly lower expression relative to rats in the other groups (box plot diagram, right). (C) Relative BDNF expression in the Steel-Dwass test: rats in the 90- and 105-week-old groups had significantly higher expression relative to those in other groups (boxplot diagram, right). In the chemiluminescence image of BDNF (left), the signal bands of the 90- and 105-week-old rats are significantly darker than those of the other three age groups, indicating higher relative expression. (D) Relative IL6 expression in the Steel-Dwass test: rats after 70 weeks of age showed significantly higher IL6 expression (box plot diagram, right). Moreover, the chemiluminescence image of IL6 (left) shows that the signal band became progressively darker with increasing age. The gray dots on the right side of the box-and-whisker plots indicate the dot plot for each group. $^{**}$P<0.01 vs. 20-week-old group; $^{*}$P<0.01 vs. 50-week-old group; $^{‡}$P<0.01 vs. 70-week-old group; $^{\#}$P<0.05 vs. 20-week-old group; $^{§}$P<0.05 vs. 50-week-old group; and $^{†}$P<0.05 vs. 70-week-old group. ER, endoplasmic reticulum; IL, interleukin; BiP/GRP78, immunoglobulin heavy chain binding protein or glucose regulatory protein 78; PDI, protein disulfide isomerase; BDNF, brain-derived neurotrophic factor.

theory, a transition from type IIb to type I fibers was observed in the fast-twitch dominant plantaris muscles of middle-aged rats (~58-67 weeks old), without muscle atrophy in the aforementioned experiments by Tamaki *et al* (35). This indicates that qualitative decline had begun even in middle-aged rats without typical sarcopenia symptoms as follows: shortening and relaxing velocity of twitch, impaired recruitment of motor units at high stimulus frequencies, and easy fatigability of the neuromuscular junction. Similarly, no loss of muscle mass was observed in the middle-aged rats (70 weeks old) in the present study, but it is possible that a decline in quality had begun and was progressing gradually.

The expression of XBP1s, which promotes UPS and autophagy, was significantly decreased in rats in the 105-week-old group relative to those in the other four groups, with no significant differences between the other four groups. Focusing on median values, rats in the 50-week-old group (median 2.5) showed a peak in XBP1s expression, followed by slight decreases at 70 (median 1.22) and 90 (median 1.1) weeks, and a significant decrease at 105 weeks (median 0.28). Effect sizes (Cohen's d) between the 50- and 70-week-old groups and between the 50- and 90-week-old groups (0.88 and 0.82, respectively) were large. This indicated that XBP1s expression peaked at 50 weeks and decreased with aging. XBP1s increases the levels of degraded proteins to promote UPS when unfolded proteins accumulate in the ER (38,39). Additionally, the accumulation of unfolded proteins in the ER also induces the expression of ER chaperones that correct the conformation of aberrant proteins (40). These findings suggest that degraded proteins accumulate in the tibial nerve of the 50-week-old rats in this study and actively induce both UPS degradation and repair by ER chaperones; this is accompanied by increased expression of XBP1s for their processing. In a histological analysis of the tibial nerve in six groups of mice (6, 9, 12, 16 and 22 months of age and a mixed age of 27 + 33 months), myelin sheath thickness of MFs peaked at 12 months (48 weeks) and decreased thereafter, while axon perimeter length peaked at 16 months (64 weeks) and decreased thereafter (16). This suggests that the myelin sheath began to atrophy at as early as 48 weeks in the aforementioned study. Similarly, myelin sheath degeneration may have been initiated in the tibial nerves of 50-week-old rats in the present study, and XBP1s expression may have increased to facilitate the repair and degradation of degenerated proteins. By contrast, XBP1s expression levels decreased after 70 weeks of age. Reduced myelin clearance, axonal regeneration and macrophage recruitment, and delayed motor recovery have been reported after sciatic nerve injury to XBP1-deficient mice in the nervous system (41). Mouse with knockout of ER stress sensor IRE1 (Inositol requiring 1), which is upstream of XBP1s (42), show accelerated age-related cognitive decline. Reduced XBP1s expression in the peripheral and central nervous system impairs the ER stress response and accelerates neurodegeneration. In the present study, neurodegeneration may have been initiated between 50 and 70 weeks of age, as XBP1s expression decreased after 70 weeks.

Expression of eIF2α increased markedly in the 90- and 105-week-old rats relative to those in the other three groups. Increased and/or activated eIF2α upregulates the translation of certain mRNAs, such as activating transcription factor 4, which regulates apoptosis-related gene expression, while inhibiting overall protein translation as an ER stress response (43). Furthermore, eIF2α activation depends on the activation of PKR-like ER kinase, a stress sensor located on the ER membrane that is activated when degraded proteins accumulate in the ER (42,44). Similar to these findings, the marked increase in eIF2α expression after 90 weeks of age in the present study suggests that inhibition of new protein biosynthesis and promotion of apoptosis may have increased. There was no difference between eIF2α expression in rats in the 50- and 70-week-old groups, but a moderate effect size (Cohen's d=0.64) was observed (data not shown). This suggested that ER stress may begin to increase gradually between 50 and 70 weeks of age. In agreement with this, Casp3, which acts downstream of apoptosis, showed a marked increase after 70 weeks of age (Fig. 1D), suggesting that accumulation of denatured proteins and degradation failure may progress between 50 and 70 weeks of age.

Becn1 expression was significantly reduced in the 105-week-old rats relative to that in the 20- and 50-week-old rats. Becn1 expression in the 70- and 90-week-old rats also tended to be lower than that in the 50-week-old rats and higher than that in the 105-week-old rats. Becn1 is associated with autophagy induction (45), and decreased Becn1 expression suppresses ER stress-induced autophagy (46). In mouse (45) and human (46) models of Alzheimer's disease, Becn1 expression was shown to decrease with age. While these findings may not be directly related to peripheral nerve tissues, reduced Becn1 expression in tibial nerves after 50 weeks of age suggests that autophagy progressively declines, resulting in the gradual accumulation of degenerative proteins. Experiments involving 293 cells with Becn1 knockdown have been shown to upregulate c-Jun N-terminal kinase (JNK1) protein expression, associated with apoptosis induction (46). Consistent with this finding, Becn1 expression in the present study decreased after 50 weeks of age, while the apoptosis-related factor Casp3 progressively increased (Fig. 1D), suggesting that aging promotes apoptosis.

Casp3 expression was markedly increased in rats in the 70-week and older age groups relative to those in the younger groups and progressively increased after 70 weeks of age. Apoptosis is induced when the degradation of denatured proteins by UPS and autophagy is reduced, resulting in prolonged accumulation of denatured proteins in the ER. ER stress-related apoptosis signaling is mainly mediated by the activation of the JNK1 (47), nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) (47,48) and Caspase 12 (Casp12) pathways (47,49,50) via the activity of IRE1 receptors present on the ER membrane. In addition, apoptosis is induced by the activation of the growth arrest and leucine zipper transcription factor, DNA damage inducible gene 153 (GADD153, also called CHOP) pathway (48) via the activities of activating transcription factor 6 and protein kinase RNA-like ER kinase receptors. Casp3 functions downstream of the caspase 12 pathway aforementioned, where calpain activity associated with increased ER stress converts inactive Pro-Casp12 to active Casp12. This is subsequently transferred from Caspase 9 activity to Casp3 activity (47,49-51). Increased Casp3 expression in the present study is expected to have been initiated between 50 and 70 weeks of age and appears to be associated with changes in XBP1s, eIF2α and Becn1 expression levels. Besides

the caspase pathway, the aforementioned, JNK1 pathway may also be promoted. Activation of the NF-κB and JNK1 pathways induces the transcription of inflammatory response-related molecules (52). Expression of IL6, an inflammatory cytokine assessed in the present study, showed a marked increase in rats after 70 weeks of age. In cultured astrocytes, treatment with the ER stress inducers thapsigargin or tunicamycin resulted in a marked increase in IL6 expression via the activation of the JNK1 pathway (53). These findings indicate that the activation of apoptotic signaling simultaneously promotes the expression of inflammatory agents. Thus, Casp3 and IL6 expression may have been similarly upregulated from 50 and 70 weeks of age in the present study.

BiP/GRP78 and PDI expression decreased progressively after 70 weeks of age. Under ER stress, BiP/GRP78 binds to unfolded proteins to prevent optimum refolding and aggregate formation, and PDI catalyzes disulfide bond formation and isomerization of secreted proteins (54-56). Besides its role in refolding denatured proteins (molecular chaperone), GRP78 promotes PDI to maintain the thiol groups of denatured proteins in a disulfide-eligible form (57). Furthermore, PDI receives electrons from the denatured protein substrate and reduces the S-S bond to a thiol group, thereby oxidizing the thiol to S-S bond in the target protein (58). Thus, both BiP/GRP78 and PDI cooperate to repair misfolded proteins. Furthermore, GRP78 binds to the IRE1 receptor and remains inactivated when not under ER stress. However, the accumulation of denatured proteins causes GRP78 to dissociate from the IRE1 receptor and activate IRE1. Activated IRE1 induces the generation of XBP1s, which promote PDI transcription (40,42). Therefore, the expression of PDI and XBP1s may regulate the signaling cascade. Consistent with this, XBP1s expression was observed to decrease in the tibial nerves of rats in the present study after 70 weeks of age (Fig. 1A). This suggests that age-related dysfunction of IRE1 signaling is more likely to occur after 70 weeks of age (or between 50 and 70 weeks of age), with both XBP1s and PDI expression showing similar declines.

BDNF expression increased progressively in rats after 70 weeks of age. BDNF promotes MF repair and preservation (59,60) and is known to be involved in the inhibition of ER stress-induced apoptosis (61,62). BDNF expression trends in the present study suggest that neurodegeneration increases markedly after 70 weeks of age, indicating increased nerve fiber repair. Similarly, a histological study of age-related changes in rat tibial nerves reported a marked progression of axonal and myelin sheath degeneration after 70 weeks of age (15). Therefore, BDNF expression may increase to promote repair after 70 weeks of age, when histological manifestations of degeneration are apparent. With respect to neurodegeneration, reactive oxygen species (ROS)-induced oxidative stress is associated with peripheral nerve degeneration and related diseases (63,64). ROS are formed in mitochondria during adenosine triphosphate production and cause damage to carbohydrates, lipids, proteins and nucleic acids (65,66). Increased cell apoptotic activity with aging is associated with increased neural concentrations of ROS (67,68). After 70 weeks of age, tibial nerve fibers are exacerbated by oxidative stress-associated damage, which may promote apoptosis. Conversely, BDNF inhibits the Casp12 pathway (apoptosis induction) via the activation of PI3K (61). As BDNF in the present study increased progressively after 70 weeks of age, it may have a role in promoting nerve fiber repair and inhibiting apoptosis. However, it is contradictory that the expression of Casp3, which functions downstream of the apoptotic pathway (Casp12 cascade), increased at ~70 weeks of age. Considering the aforementioned histological progression of tibial nerve MF degeneration after 70 weeks of age (15), the promotion of apoptosis may be dominant over the promotion of repair by BDNF, resulting in an imbalance between these two antagonistic pathways.

Regarding IL6 expression, the 90- and 105-week-old rats showed a significant increase relative to those in the 50- and 20-week-old groups. There was no significant difference between the 70- and 50-week-old groups, but the effect size (Cohen's d=0.98) (data not shown) was large, indicating a substantial increase at 70 weeks and thereafter. IL6 induces inflammatory responses that induce neutrophil and macrophage migration under ER stress conditions, and its biosynthesis is ROS-inducible (69). It is also known that XBP1 is a regulator of IL6 production, which is essential for plasma cell differentiation, survival and immunoglobulin synthesis, in addition to unfolding protein repair and UPS induction (70,71). These findings suggest that IL6 expression is upregulated as XBP1 expression increases under ER stress. The present study reveals contradictory results: the expression of spliced XBP1 mRNAs (XBP1s) decreased after 70 weeks of age, whereas IL6 expression increased after 70 weeks of age. As discussed for Casp3 earlier, promotion of the JNK1 pathway may be involved in the regulation of IL6 expression (52). Therefore, it is suggested that increased IL6 expression after 70 weeks in the present study was not affected by the regulation of XBP1 (or XBP1s) expression and was caused by the promotion of apoptosis through increased Casp3 expression. Transient injection of IL6 in rats decreased myofibrillar protein by 17% and total muscle protein by 9%. A standard deviation increase in IL6 expression levels in older patients decreased grip strength by 1.12-2.37 kg, suggesting that increased IL6 expression may also cause atrophy of peripheral nerve fiber axons and myelin sheaths (72,73). Mitochondrial dysfunction is also associated with increased apoptosis with aging. Age-related mitochondrial dysfunction leads to increased ROS, resulting in increased accumulation of degenerative proteins and DNA damage (74). This could be a reason for the tendency of degenerated proteins to accumulate after 70 weeks in the present study. Hence, it is suggested that the promotion of apoptosis by the accumulation of denatured proteins induces increased IL6 expression. Furthermore, both BDNF and IL6 expression increased progressively after 50 weeks. Previous studies suggested that BDNF and IL6 expression levels increase in acute neuroinflammation (75,76). However, in chronic inflammation, IL6 expression is maintained or increased, whereas BDNF expression levels are reduced (77), which is not in agreement with the results of the present study. Although there is little direct evidence to resolve this discrepancy, there are factors to consider. For instance, BDNF levels were reported to be low when the ratio of inflammatory cytokines to the anti-inflammatory cytokine IL6 in the prefrontal cortex was significantly higher than normal (77). That is, anti-inflammatory cytokines may be involved in the increased expression of BDNF. Therefore, it may be necessary to consider the

expression of anti-inflammatory cytokines in addition to the inflammatory cytokine IL6 in the present study.

Oxidative stress, including the generation of ROS and free radicals, is widely recognized as an important contributor to age-related neuronal degeneration and death (29,68). In spinal neurons, for instance, the activity of proteasomes-a critical defense mechanism that mitigates oxidative damage to DNA, proteins and lipids-declines significantly with advancing age. This decrease in proteasome function has been reported to be associated with motor neuron degeneration in aging rats (30). Similarly, in animal models characterized by increased oxidative stress, motor dysfunction develops early, and structural damage to axons, myelin sheaths, and neuronal cell bodies is observed by middle age. This accelerated degeneration is similar to the changes associated with normal aging but occurs at an earlier stage (31). In the present study, the rats were subjected to a lifetime of physical inactivity, which may have resulted in a marked increase in ROS production as early as 70 weeks of age and after. ROS also interact with the ER stress pathway, creating a feedback loop that exacerbates cellular damage. ROS production is associated with the activation of ER stress via pathways such as the UPR, which amplifies oxidative stress by disrupting redox homeostasis in the ER lumen (78). This bidirectional relationship suggests that oxidative and ER stress act synergistically to promote age-related neuronal apoptosis and inflammation. Given the interplay between oxidative stress and neuroprotective mechanisms, it is plausible that increased ROS levels contribute to the progressive regression of neurons in the central and peripheral nervous systems. Moreover, the impact of reduced antioxidant defenses, such as proteasome activity, likely extends to motor MFs in peripheral nerves, exacerbating age-related degenerative changes.

Overall, it was observed in the present study that expression levels of ER stress-related degradation proteins XBP1s, eIF2α, Becn1 and Casp3 begin to change between 50 and 70 weeks of age in rats. XBP1s and Becn1, which are associated with UPS and autophagy in response to ER stress, respectively, are significantly reduced after 50 weeks of age. This suggests a decline in their degradation systems. Hence, it is likely that after 50 weeks of age, new protein synthesis was suppressed (increased eIF2α expression) and apoptosis was promoted (increased Casp3 expression) along with the accumulation of denatured proteins. Additionally, expression levels of ER stress-related repair proteins BiP/GRP78, PDI, BDNF and IL6 changed between 50 and 70 weeks of age. BiP/GRP78 expression remained unchanged between 50 and 70 weeks of age, whereas that of PDI decreased. Furthermore, after 70 weeks of age, both BiP/GRP78 and PDI expression decreased remarkably with age. Thus, the repair function begins to decline between 50 and 70 weeks of age and continues to decline with age. Previous studies have shown that oxidative stress and free radicals may be associated with age-related neuronal regression (29,68). The activity of proteasomes, which protect proteins, DNA and lipids in spinal cord neurons from oxidative stress, was markedly reduced in rats after 12 months (48 weeks), mediating motor neuron degeneration (30). Presumably, the regression of spinal cord neurons after ~50 weeks involves reduced activity of neuroprotective factors such as proteasomes, oxidative stress and increased free radicals, which may also affect peripheral nerve MFs. However, BDNF gradually increased after 50 weeks of age, compensating for the decline in refolding function. Apoptosis (Casp3) was also promoted at the same time after 70 weeks of age, suggesting that apoptosis may become predominant with aging. Mitochondrial dysfunction occurred between 50 and 70 weeks of age, resulting in increased oxidative stress, accumulation of denatured proteins, increased IL6 expression and apoptosis.

There are certain limitations to the present study. First, it was not possible to have homogeneity in the numbers of rats in each age group. This is because after 90 weeks, a few more rats than expected succumbed before reaching 105 weeks of age, resulting in a few less rats in the 105-week-old group, relative to the other groups. The possibility that the bias introduced due to this heterogeneity in sample size may have reduced the power to detect variance and between-group differences, cannot be denied. Therefore, to resolve this problem as much as possible, effect size tests were also performed as appropriate. Second, male rats were exclusively used to minimize the confounding effects of hormonal fluctuations associated with the estrous cycle in females. Previous studies suggest that hormonal factors, such as estrogen, may play a neuroprotective role in female rats, potentially mitigating age-related neurodegeneration and associated inflammation (79,80). The absence of such protective effects in male rats might have amplified the observed changes in the present study. Therefore, it is plausible that female rats might exhibit different patterns of neurodegeneration and inflammatory responses with aging. Future research should address these limitations by including rats of both sexes to fully explore the interplay between sex hormones and age-related changes. Third, focus was addressed on changes in the expression levels of ER stress-related degradation proteins, repair proteins, and the inflammatory cytokine IL6 with aging. However, further determination of ER stress by assessing the concentrations of ROS and the total amount of degenerated proteins affecting peripheral nerve degeneration was not performed. Because the present study did not directly assess ROS levels, future research should explore this relationship in detail to allow for an improved understanding of the combined effects of oxidative stress and ER stress on peripheral nerve degeneration during aging. The inclusion of ROS measurements in subsequent experiments will provide valuable insights into the molecular pathways underlying these phenomena. Additionally, histological and morphological analysis of the aging tibial nerve was not performed. Biochemical, histological and morphological changes associated with aging may not develop simultaneously. Rather, histological changes may be delayed. Therefore, the differences in the time scales between biochemical, histological and morphological changes should be clarified in future studies and applied to prevention and treatment strategies. Finally, it is important to acknowledge the limitations of the present study regarding the use of Casp3 and IL6 as biomarkers for apoptosis and induction of inflammation, respectively. These markers are not specific to ER stress and may be affected by other aging-related pathways. This limitation may affect the specificity of the current findings on ER stress-related apoptosis and inflammation. Future studies will address these

limitations by incorporating biomarkers such as family with sequence similarity 134, member B (FAM134B) and tumor necrosis factor-α (TNF-α), which are specific biomarkers in the induction of ER stress response-related apoptosis and inflammation, respectively. FAM134B, a member of the reticulon family, has been identified as a crucial mediator of ER-phagy, a selective autophagic process that regulates ER turnover and maintains ER homeostasis. Dysregulation of FAM134B has been implicated in neurodegenerative diseases and peripheral neuropathy due to its role in ER stress-induced apoptosis (81,82). Assessing FAM134B expression would provide a more specific indication of ER stress-related apoptotic mechanisms in the peripheral nervous system. Similarly, TNF-α is a pro-inflammatory cytokine that plays a central role in mediating inflammation during ER stress. TNF-α is known to be upregulated in response to the UPR signaling pathway, specifically through the activation of the PERK and IRE1 pathways, which are key components of ER stress (83). The inclusion of TNF-α as a marker could enhance the specificity of our evaluation of ER stress-related inflammation. Moreover, analyzing the expression of FAM134B and TNF-α will increase the specificity of studies on the ER stress response in peripheral nerves. Future investigations will be designed to include FAM134B and TNF-α as specific markers for ER stress-induced apoptosis and inflammation, thereby addressing the limitations of our current approach.

In conclusion, in the present study, changes in expression levels of ER stress-related UPS and autophagy, apoptosis, repair, and inflammatory cytokine proteins were examined to demonstrate signs of age-related neurodegeneration in rats. It was observed that UPS and autophagy declined after 50 weeks of age, followed by the promotion of apoptosis. Repair function decreased after 50 weeks of age, whereas the expression of the inflammatory cytokine IL6 increased after 50 weeks of age. Future research should examine the inhibitory effects of pre- and post-50-week regular aerobic exercise (treatment) interventions on age-related neurodegeneration in terms of the ER stress response.

## Acknowledgements

Not applicable.

## Funding

The present study was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (https://kaken.nii.ac.jp; grant no. 20K19694).

## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

MS conceived the study design and planned the study. MS, KS, SS, AY and KY performed statistical analyses and prepared figures and tables. MS, WI, KN, SM and RM managed the animal breeding and conducted the experiments. MS coordinated the experiments and wrote the manuscript. All authors read and approved the final version of the manuscript. MS, KS, SS, AY and KY confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Kyoto Tachibana University (approval no. 19-10; Kyoto, Japan) and was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (National Research Council, 1996).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Beaudart C, Zaaria M, Pasleau F, Reginster JY and Bruyère O: Health outcomes of sarcopenia: A systematic review and meta-analysis. PLoS One 12: e0169548, 2017.
2. Kelley GA and Kelley KS: Is sarcopenia associated with an increased risk of all-cause mortality and functional disability? Exp Gerontol 96: 100-103, 2017.
3. Chen X, Cao M, Liu M, Liu S, Zhao Z and Chen H: Association between sarcopenia and cognitive impairment in the older people: A Meta-analysis. Eur Geriatr Med 13: 771-787, 2022.
4. Zhang X, Huang P, Dou Q, Wang C, Zhang W, Yang Y, Wang J, Xie X, Zhou J and Zeng Y: Falls among older adults with sarcopenia dwelling in nursing home or community: A meta-analysis. Clin Nutr 39: 33-39, 2020.
5. Landi F, Liperoti R, Russo A, Giovannini S, Tosato M, Capoluongo E, Bernabei R and Onder G: Sarcopenia as a risk factor for falls in elderly individuals: Results from the ilSIRENTE study. Clin Nutr 31: 652-658, 2012.
6. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel JP, Rolland Y, Schneider SM, *et al*: Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. Age Ageing 39: 412-423, 2010.
7. Su YC, Chang SF and Tsai HC: The relationship between sarcopenia and injury events: A systematic review and Meta-analysis of 98,754 Older Adults. J Clin Med 11: 6474, 2022.
8. Yeung SSY, Reijnierse EM, Pham VK, Trappenburg MC, Lim WK, Meskers CGM and Maier AB: Sarcopenia and its association with falls and fractures in older adults: A systematic review and meta-analysis. J Cachexia Sarcopenia Muscle 10: 485-500, 2019.
9. Verdú E, Ceballos D, Vilches JJ and Navarro X: Influence of aging on peripheral nerve function and regeneration. J Peripher Nerv Syst 5: 191-208, 2000.
10. Coletti C, Acosta GF, Keslacy S and Coletti D: Exercise-mediated reinnervation of skeletal muscle in elderly people: An update. Eur J Transl Myol 32: 1-11, 2022.
11. Liu J, Zhang B, Lei H, Feng Z, Liu J, Hsu AL and Xu XZ: Functional aging in the nervous system contributes to age-dependent motor activity decline in C. elegans. Cell Metab 18: 392-402, 2013.
12. Nagashima K and Oota K: A histopathological study of the human spinal ganglia. 1. Normal variations in aging. Acta Pathol Jpn 24: 333-344, 1974.
13. O'Sullivan DJ and Swallow M: The fibre size and content of the radial and sural nerves. J Neurol Neurosurg Psychiatry 31: 464-470, 1968.
14. Bergman E and Ulfhake B: Loss of primary sensory neurons in the very old rat: Neuron number estimates using the disector method and confocal optical sectioning. J Comp Neurol 396: 211-222, 1998.

15. Sakita M, Murakami S, Nonaka K, Sakamoto R, Saito T, Isobe W and Kumagai S: Different patterns in age-related morphometric alteration of myelinated fibers and capillaries of the tibial nerve: A longitudinal study in normal rats. J Anat 236: 1101-1111, 2020.
16. Ceballos D, Cuadras J, Verdu E and Navarro X: Morphometric and ultrastructural changes with ageing in mouse peripheral nerve. J Anat 195: 563-576, 1999.
17. Bouche P, Cattelin F, Saint-Jean O, Léger JM, Queslati S, Guez D, Moulonguet A, Brault Y, Aquino JP and Simunek P: Clinical and electrophysiological study of the peripheral nervous system in the elderly. J Neurol 240: 263-268, 1993.
18. Verdú E, Butí M and Navarro X: Functional changes of the peripheral nervous system with aging in the mouse. Neurobiol Aging 17: 73-77, 1996.
19. Ciechanover A: Intracellular protein degradation: From a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. Cell Death Differ 12: 1178-1190, 2005.
20. Aman Y, Schmauck-Medina T, Hansen M, Morimoto RI, Simon AK, Bjedov I, Palikaras K, Simonsen A, Johansen T and Tavernarakis N: Autophagy in healthy aging and disease. Nat Aging 1: 634-650, 2021.
21. Rajawat YS, Hilioti Z and Bossis I: Aging: Central role for autophagy and the lysosomal degradative system. Ageing Res Rev 8: 199-213, 2009.
22. Amm I, Sommer T and Wolf DH: Protein quality control and elimination of protein waste: The role of the ubiquitin-proteasome system. Biochim Biophys Acta 1843: 182-196, 2014.
23. Guo J, Huang X, Dou L, Yan M, Shen T, Tang W and Li J: Aging and aging-related diseases: From molecular mechanisms to interventions and treatments. Signal Transduct Target Ther 7: 391, 2022.
24. Perner C and Krüger E: Endoplasmic reticulum stress and its role in homeostasis and immunity of central and peripheral neurons. Front Immunol 13: 859703, 2022.
25. Shinno K, Miura Y, Iijima KM, Suzuki E and Ando K: Axonal distribution of mitochondria maintains neuronal autophagy during aging via eIF2β. bioRxiv 2024: 576435, 2024.
26. Cuervo AM and Dice JF: How do intracellular proteolytic systems change with age? Front Biosci 3: d25-d43, 1998.
27. Hughes AL and Gottschling DE: An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. Nature 492: 261-265, 2012.
28. Leidal AM, Levine B and Debnath J: Autophagy and the cell biology of age-related disease. Nat Cell Biol 20: 1338-1348, 2018.
29. Thrasivoulou C, Soubeyre V, Ridha H, Giuliani D, Giaroni C, Michael GJ, Saffrey MJ and Cowen T: Reactive oxygen species, dietary restriction and neurotrophic factors in age-related loss of myenteric neurons. Aging Cell 5: 247-257, 2006.
30. Keller JN, Huang FF and Markesbery WR: Decreased levels of proteasome activity and proteasome expression in aging spinal cord. Neuroscience 98: 149-156, 2000.
31. Sims-Robinson C, Hur J, Hayes JM, Dauch JR, Keller PJ, Brooks SV and Feldman EL: The role of oxidative stress in nervous system aging. PLoS One 8: e68011, 2013.
32. Eaton SL, Roche SL, Llavero Hurtado M, Oldknow KJ, Farquharson C, Gillingwater TH and Wishart TM: Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. PLoS One 8: e72457, 2013.
33. Moritz CP: Tubulin or not tubulin: Heading toward total protein staining as loading control in western blots. Proteomics: 17, 2017 doi: 10.1002/pmic.201600189.
34. Nistiar F, Racz O, Lukacinova A, Hubkova B, Novakova J, Lovasova E and Sedlakova E: Age dependency on some physiological and biochemical parameters of male Wistar rats in controlled environment. J Environ Sci Health A Tox Hazard Subst Environ Eng 47: 1224-1233, 2012.
35. Tamaki T, Hirata M and Uchiyama Y: Qualitative alteration of peripheral motor system begins prior to appearance of typical sarcopenia syndrome in middle-aged rats. Front Aging Neurosci 6: 296, 2014.
36. Gao Q, Hu K, Yan C, Zhao B, Mei F, Chen F, Zhao L, Shang Y, Ma Y and Ma B: Associated factors of sarcopenia in community-Dwelling older adults: A systematic review and Meta-analysis. Nutrients 13: 4291, 2021.
37. Cruz-Jentoft AJ and Sayer AA: Sarcopenia. Lancet 393: 2636-2646, 2019.
38. Hollien J and Weissman JS: Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science 313: 104-107, 2006.
39. Hollien J, Lin JH, Li H, Stevens N, Walter P and Weissman JS: Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J Cell Biol 186: 323-331, 2009.
40. Park SM, Kang TI and So JS: Roles of XBP1s in transcriptional regulation of target genes. Biomedicines 9: 791, 2021.
41. Oñate M, Catenaccio A, Martínez G, Armentano D, Parsons G, Kerr B, Hetz C and Court FA: Activation of the unfolded protein response promotes axonal regeneration after peripheral nerve injury. Sci Rep 6: 21709, 2016.
42. Cabral-Miranda F, Tamburini G, Martinez G, Ardiles AO, Medinas DB, Gerakis Y, Hung MD, Vidal R, Fuentealba M, Miedema T, *et al*: Unfolded protein response IRE1/XBP1 signaling is required for healthy mammalian brain aging. EMBO J 41: e111952, 2022.
43. Costa-Mattioli M and Walter P: The integrated stress response: From mechanism to disease. Science 368: 2020.
44. Kashiwagi K, Yokoyama T, Nishimoto M, Takahashi M, Sakamoto A, Yonemochi M, Shirouzu M and Ito T: Structural basis for eIF2B inhibition in integrated stress response. Science 364: 495-499, 2019.
45. Rashid HO, Yadav RK, Kim HR and Chae HJ: ER stress: Autophagy induction, inhibition and selection. Autophagy 11: 1956-1977, 2015.
46. Li J, Ni M, Lee B, Barron E, Hinton DR and Lee AS: The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ 15: 1460-1471, 2008.
47. Smith MI and Deshmukh M: Endoplasmic reticulum stress-induced apoptosis requires bax for commitment and Apaf-1 for execution in primary neurons. Cell Death Differ 14: 1011-1019, 2007.
48. Kaufman RJ: Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. Genes Dev 13: 1211-1233, 1999.
49. Morishima N, Nakanishi K, Takenouchi H, Shibata T and Yasuhiko Y: An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. J Biol Chem 277: 34287-34294, 2002.
50. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S: A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391: 43-50, 1998.
51. Rasheva VI and Domingos PM: Cellular responses to endoplasmic reticulum stress and apoptosis. Apoptosis 14: 996-1007, 2009.
52. Chaudhari N, Talwar P, Parimisetty A, Lefebvre d'Hellencourt C and Ravanan P: A molecular web: Endoplasmic reticulum stress, inflammation, and oxidative stress. Front Cell Neurosci 8: 213, 2014.
53. Sanchez CL, Sims SG, Nowery JD and Meares GP: Endoplasmic reticulum stress differentially modulates the IL-6 family of cytokines in murine astrocytes and macrophages. Sci Rep 9: 14931, 2019.
54. Brocchieri L, Conway de Macario E and Macario AJ: hsp70 genes in the human genome: Conservation and differentiation patterns predict a wide array of overlapping and specialized functions. BMC Evol Biol 8: 19, 2008.
55. Ibrahim IM, Abdelmalek DH and Elfiky AA: GRP78: A cell's response to stress. Life Sci 226: 156-163, 2019.
56. Ellgaard L and Ruddock LW: The human protein disulphide isomerase family: Substrate interactions and functional properties. EMBO Rep 6: 28-32, 2005.
57. Mayer M, Kies U, Kammermeier R and Buchner J: BiP and PDI cooperate in the oxidative folding of antibodies in vitro. J Biol Chem 275: 29421-29425, 2000.
58. Grek C and Townsend DM: Protein disulfide isomerase superfamily in disease and the regulation of apoptosis. Endoplasmic Reticulum Stress Dis 1: 4-17, 2014.
59. Helan M, Aravamudan B, Hartman WR, Thompson MA, Johnson BD, Pabelick CM and Prakash YS: BDNF secretion by human pulmonary artery endothelial cells in response to hypoxia. J Mol Cell Cardiol 68: 89-97, 2014.
60. Wilhelm JC, Xu M, Cucoranu D, Chmielewski S, Holmes T, Lau KS, Bassell GJ and English AW: Cooperative roles of BDNF expression in neurons and Schwann cells are modulated by exercise to facilitate nerve regeneration. J Neurosci 32: 5002-5009, 2012.

61. Shimoke K, Utsumi T, Kishi S, Nishimura M, Sasaya H, Kudo M and Ikeuchi T: Prevention of endoplasmic reticulum stress-induced cell death by brain-derived neurotrophic factor in cultured cerebral cortical neurons. Brain Res 1028: 105-111, 2004.
62. Bhaskar PT and Hay N: The two TORCs and Akt. Dev Cell 12: 487-502, 2007.
63. Scott TL, Rangaswamy S, Wicker CA and Izumi T: Repair of oxidative DNA damage and cancer: Recent progress in DNA base excision repair. Antioxid Redox Signal 20: 708-726, 2014.
64. Fukui K: Reactive oxygen species induce neurite degeneration before induction of cell death. J Clin Biochem Nutr 59: 155-159, 2016.
65. Ma Q: Transcriptional responses to oxidative stress: Pathological and toxicological implications. Pharmacol Ther 125: 376-393, 2010.
66. Hong Y, Boiti A, Vallone D and Foulkes NS: Reactive oxygen species signaling and oxidative stress: transcriptional regulation and evolution. Antioxidants (Basel) 13: 312, 2024.
67. Shokouhi G, Tubbs RS, Shoja MM, Roshangar L, Mesgari M, Ghorbanihaghjo A, Ahmadi N, Sheikhzadeh F and Rad JS: The effects of aerobic exercise training on the age-related lipid peroxidation, Schwann cell apoptosis and ultrastructural changes in the sciatic nerve of rats. Life Sci 82: 840-846, 2008.
68. Wickens AP: Ageing and the free radical theory. Respir Physiol 128: 379-391, 2001.
69. Haddad JJ, Saadé NE and Safieh-Garabedian B: Redox regulation of TNF-alpha biosynthesis: augmentation by irreversible inhibition of gamma-glutamylcysteine synthetase and the involvement of an IkappaB-alpha/NF-kappaB-independent pathway in alveolar epithelial cells. Cell Signal 14: 211-218, 2002.
70. Iwakoshi NN, Lee AH, Vallabhajosyula P, Otipoby KL, Rajewsky K and Glimcher LH: Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. Nat Immunol 4: 321-329, 2003.
71. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, Friend D, Grusby MJ, Alt F and Glimcher LH: Plasma cell differentiation requires the transcription factor XBP-1. Nature 412: 300-307, 2001.
72. Haddad F, Zaldivar F, Cooper DM and Adams GR: IL-6-induced skeletal muscle atrophy. J Appl Physiol (1985) 98: 911-917, 2005.
73. Visser M, Pahor M, Taaffe DR, Goodpaster BH, Simonsick EM, Newman AB, Nevitt M and Harris TB: Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: The Health ABC Study. J Gerontol A Biol Sci Med Sci 57: M326-M332, 2002.
74. Lima T, Li TY, Mottis A and Auwerx J: Pleiotropic effects of mitochondria in aging. Nat Aging 2: 199-213, 2022.
75. Xu S, Ito A, Wang T, Kawai H, Aoyama T and Kuroki H: Ultrasound therapy of injury site modulates gene and protein expressions in the dorsal root ganglion in a sciatic nerve crush injury rat model. Ultrasound Med Biol 48: 2502-2511, 2022.
76. Lin G, Zhang H, Sun F, Lu Z, Reed-Maldonado A, Lee YC, Wang G, Banie L and Lue TF: Brain-derived neurotrophic factor promotes nerve regeneration by activating the JAK/STAT pathway in Schwann cells. Transl Androl Urolo 5: 167-175, 2016.
77. Xie ZM, Wang XM, Xu N, Wang J, Pan W, Tang XH, Zhou ZQ, Hashimoto K and Yang JJ: Alterations in the inflammatory cytokines and brain-derived neurotrophic factor contribute to depression-like phenotype after spared nerve injury: Improvement by ketamine. Sci Rep 7: 3124, 2017.
78. Malhotra JD and Kaufman RJ: Endoplasmic reticulum stress and oxidative stress: A vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277-2293, 2007.
79. Shindo S, Chen SH, Gotoh S, Yokobori K, Hu H, Ray M, Moore R, Nagata K, Martinez J, Hong JS and Negishi M: Estrogen receptor α phosphorylated at Ser216 confers inflammatory function to mouse microglia. Cell Commun Signal 18: 117, 2020.
80. Villa A, Vegeto E, Poletti A and Maggi A: Estrogens, neuroinflammation, and neurodegeneration. Endocr Rev 37: 372-402, 2016.
81. Khaminets A, Heinrich T, Mari M, Grumati P, Huebner AK, Akutsu M, Liebmann L, Stolz A, Nietzsche S, Koch N, *et al*: Regulation of endoplasmic reticulum turnover by selective autophagy. Nature 522: 354-358, 2015.
82. Cinque L, De Leonibus C, Iavazzo M, Krahmer N, Intartaglia D, Salierno FG, De Cegli R, Di Malta C, Svelto M, Lanzara C, *et al*: MiT/TFE factors control ER-phagy via transcriptional regulation of FAM134B. EMBO J 39: e105696, 2020.
83. Zhang K and Kaufman RJ: From endoplasmic-reticulum stress to the inflammatory response. Nature 454: 455-462, 2008.