Endoplasmic reticulum stress contributes to the pathogenesis of stress urinary incontinence in postmenopausal women

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Abstract

Objective: To investigate the relationship between endoplasmic reticulum stress (ERS) and the pathogenesis of stress urinary incontinence (SUI) in postmenopausal women.

Methods: Anterior vaginal wall tissue was collected from postmenopausal women with SUI and control subjects. Western blotting was performed for glucose-regulated protein (GRP78), inositol-requiring enzyme 1(IRE1), protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), C/EBP-homologous protein (CHOP), and B-cell lymphoma 2 (Bcl-2). Additionally, mRNA expression levels of PERK, activating transcription factor 4 (ATF4), and CHOP were examined by real-time polymerase chain reaction.

Results: GRP78 protein and mRNA expression levels were significantly lower in women with SUI, compared with control subjects. PERK and p-PERK expression levels were higher in women with SUI than in control subjects. However, no differences in IRE1 or ATF6 expression levels were observed in either group. Notably, higher CHOP and lower Bcl-2 protein expression levels were detected in women with SUI, compared with control subjects. Furthermore, PERK, ATF4, and CHOP mRNA expression levels were significantly higher in women with SUI than in control subjects.

Conclusions: Alterations of ERS markers in SUI suggest that ERS may be involved in the development of SUI in postmenopausal women.

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Introduction

Stress urinary incontinence (SUI) is defined as the involuntary leakage of urine that occurs when intra-abdominal pressure exceeds urethral pressure during coughing, sneezing, or physical exertion. SUI is a distressing problem that profoundly compromises health-associated quality of life.[1] As is well-known, the prevalence of SUI peaks around and after the period of menopause.[2] However, the mechanism of SUI remains unclear. Several studies[3–5] have shown that the onset of SUI is associated with injury to pelvic floor tissues, which can cause failure of pelvic homeostasis, increasing urethral hypermobility and inducing involuntary urine leakage.

Endoplasmic reticulum stress (ERS) is a major signal-transducing event involved in sensing of and response to changes in homeostasis.[6] Endoplasmic reticulum (ER) function can be severely impaired by various genetic and environmental factors. Subsequently, unfolded or misfolded proteins accumulate in the ER lumen; these changes may result in ERS. Additionally, excessive ERS elicits apoptotic signals that ultimately cause disturbances to overall homeostasis of the body.[7] ERS has been shown to play a role in the onset of degenerative diseases such as retinal degeneration, Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease.[8] Moreover, studies[3,9] have revealed that SUI is likely the result of degenerative changes in pelvic floor tissues. Thus, we speculated that the development of SUI might be associated with excessive ERS. Therefore, the present study was designed to determine the expression of relevant markers of ERS in the anterior vaginal wall, and to reveal the potential link between ERS and the pathogenesis of SUI in postmenopausal women.

Patients and methods

Ethics statement

All experiments were approved by the Ethics Review Board of Women's Hospital, Zhejiang University School of Medicine; written informed consent was obtained from all patients.

Subjects and samples

Postmenopausal patients diagnosed with SUI in accordance with the recommendations of the International Continence Society,[10] who underwent tension-free vaginal tape surgery at the Department of Gynecology, Women's Hospital, Zhejiang University School of Medicine, between July 2016 and June 2017, were included in the study. A matching number of postmenopausal women, who underwent intravaginal cystectomy for vaginal wall cyst without SUI or pelvic organ prolapse (POP), were recruited as control subjects during the same time period.

Exclusion criteria for the study were as follows: hormone replacement therapy in the last 3 months; urinary infection; estrogen-dependent disease (endometriosis,

fibroid or functional ovarian tumor); clinical POP; pre-menopause status; and/or urge incontinence. Postmenopausal women were defined as women who had experienced 12 consecutive months without menstruation after menopause. All participants enrolled in this study were diagnosed objectively by the combination of medical history, gynecological examination, urinary stress test, ultrasonography, urodynamic examination, and POP-Q test.[11]

As in our previous studies,[12,13] biopsy samples of the anterior vaginal wall, used as urethral supporting tissue in SUI, were taken 1–2 cm from the uterine cervix, including mucosa, submucosa, connective tissue, and smooth muscle. All tissue was frozen immediately at $-80°C$ for further analysis.

Cell counting

Tissue samples were fixed in 10% formalin in phosphate-buffered saline at 4°C overnight, then routinely trimmed and embedded in paraffin, and serially sectioned at 4-μm thickness. Slices were subsequently stained with hematoxylin and eosin. Cell density was counted in representative light photomicrographs.

Western blotting analysis

Tissue samples were homogenized in Radio-Immunoprecipitation Assay Lysis and Extraction Buffer (containing a protease inhibitor cocktail; Pierce, Rockford, IL, USA). The processes were performed in accordance with previous research.[14] Insoluble material was removed by centrifugation at $12,000 \times g$ at 4°C for 30 minutes. Proteins were determined by using a commercial bicinchoninic acid kit (Sigma, St. Louis, MO, USA). An equal amount of total protein (60 μg per sample) was loaded per lane and was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to poly-vinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked in 10% non-fat milk for 1 hour at 25°C, then washed three times with Tris-buffered saline-0.1% Tween-20 at 25°C (5 minutes per wash). Primary antibodies were anti-GRP78 (1:500 dilution), anti-PERK (1:500 dilution), anti-phosphorylated PERK (p-PERK; 1:500 dilution), anti-inositol-requiring enzyme 1 (IRE1; 1:500 dilution), anti- activating transcription factor 6 (ATF6; 1:500 dilution), anti-CHOP (1:500 dilution), and anti-B-cell lymphoma 2 (Bcl-2; 1:500 dilution) antibodies. All of the above antibodies were purchased from Santa Cruz Biotech (Dallas, TX, USA). An anti-beta-actin antibody (1:500 dilution; Cell Signaling, Danvers, MA, USA) was used for normalization of protein expression. The secondary antibody was a horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG (1:5000 dilution; Santa Cruz Biotech). Protein-antibody complexes were detected by enhanced chemiluminescence (Millipore, Burlington, MA, USA) and exposure to an X-Omat film (Kodak, Xiamen, China). The relative band densities were quantified by Quantity One 1-D analysis Software (version 4.6.9, Bio-Rad Laboratories, Hercules, CA, USA).

Real-time polymerase chain reaction (PCR) RNA analysis

Total mRNA was extracted from tissue samples by using the single-step method with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and quantified using spectrophotometry (NANOdrop 2000; Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed by using SYBR Green dye (Life Technologies) and the Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA,

USA), in accordance with the manufacturer's instructions. The following primers were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China): Human Chop10 (F:5′-GAACGGC TCAAGCAGGAAATC-3′, R:5′-GATTCA CCATTCGG TCAATCAGAG-3′), Human PERK (F:5′-TCTTCCTTGGGTCTGATG AT-3′, R:5′GATGTTCTTGCTGTAGTGC CG-3′), Human ATF4 (F:5′-GTCTCCGT GAGCGTCCAT-3′, R:5′-CAGAAGCCAA CTCCCATTAG-3′), Human GRP78 (F:5′-CATCACGCCGTCCTATGTCG-3′, R:5′-GGCGTCAAAGACCGTGTTCTC-3′), and Human GAPDH (F:5′-CCATGAC AACTTTGGTATCGTGGAA-3′, R:5′-GG CCATCACGCC ACAGTTTC-3′). The following real-time PCR reaction protocol was used, based on a previous study:[15] 2.5 μl dNTPs (2.5 mM each); 2.5 μl 10X PCR buffer; 1 unit of Taq polymerase; 12.5 μl SYBR Premix Ex Taq^{TM} (50 μl per reaction × 200 reactions, Takara Biotechnology, Dalian, China); 1 μl each primer; 1 μl cDNA; and double-distilled water to a final volume of 25 μl. Real-time PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of elongation (95°C for 15 s, 60°C for 30 s), and a final elongation at 72°C for 30 s. Data were normalized against GAPDH and analyzed using the comparative $2^{-\triangle\triangle Ct}$ method.

Statistical analysis

Data are presented as mean ± standard deviation. Student's t-test was used to compare variables between the two groups. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

This study included 10 patients with SUI (mean age 52.46 ± 3.77 years; body mass index (BMI) 21.91 ± 7.44 kg/m^2; parity 1.78 ± 0.56; and menopausal period 1.78 ± 0.56 years) and 10 control subjects (mean age 53.26 ± 4.83 years; BMI 22.37 ± 8.13 kg/m^2; parity 1.90 ± 0.69; and menopausal period 1.90 ± 0.69 years). No significant differences were observed in age, parity, menopausal period, or BMI; these data showed good comparability between the groups.

Cell counting

Hematoxylin and eosin staining showed disordered cell arrangement in women with SUI. The density of cells was significantly lower in the SUI group than in the control group (6.70 ± 1.34 vs. 38.5 ± 4.23; $P < 0.05$) (Figure 1).
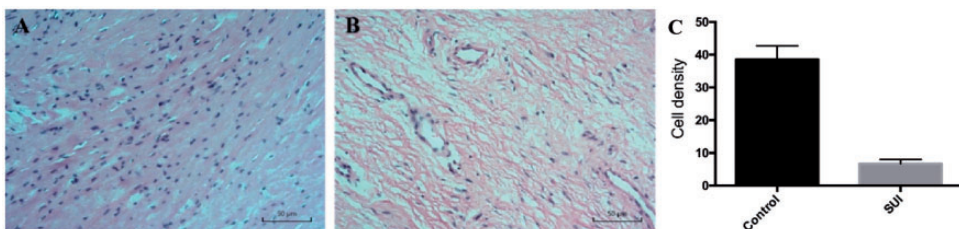

Figure 1. HE (×200) staining for smooth muscle cells and fibroblasts in anterior vaginal wall tissue. (A) Control subjects (women without SUI); (B) women with SUI. (C) Five fields of view were selected in each slice in light photomicrographs of HE (×400) and average cell density was determined. Cell density decreased significantly in women with SUI, compared with control subjects, $P < 0.05$. HE, hematoxylin and eosin; SUI, stress urinary incontinence.

Expression of relevant markers of ERS

GRP78 protein expression was significantly lower in postmenopausal women with SUI, compared with control subjects. PERK and p-PERK expression levels were higher in women with SUI, compared with control subjects ($P < 0.05$); no differences in IRE1 or ATF6 were observed in either group. The results suggested that PERK, mainly activated in responses to various types of ERS, may contribute to SUI in postmenopausal women (Figure 2).

Notably, higher CHOP and lower Bcl-2 protein expression levels were measured in women with SUI, compared with control subjects ($P < 0.05$); this implied enhanced apoptotic activity in postmenopausal women with SUI (Figure 3).

mRNA expression of the PERK-ATF4-CHOP pathway

Expression of GRP78 mRNA was lower, while PERK, ATF4, and CHOP mRNA levels were enhanced in postmenopausal women with SUI, compared with control subjects ($P < 0.05$). This suggested that the entire PERK-ATF4-CHOP pathway, active in ERS and related to apoptosis, is involved in SUI in postmenopausal women (Figure 4).

Discussion

The relationship between ERS and various types of damage has recently become an important research topic. ERS is generally involved in mechanisms of cell protection; however, excessive ERS may result in cellular damage.[16,17] Typically, severe ERS disturbs the self-protection mechanism and triggers apoptosis by a highly conserved signal transduction pathway, known as the unfolded protein response (UPR).[18] As previously reported,[19] UPR signaling is orchestrated by three different pathways, each of which is initiated by a distinct sensor anchored in the ER as a signaling
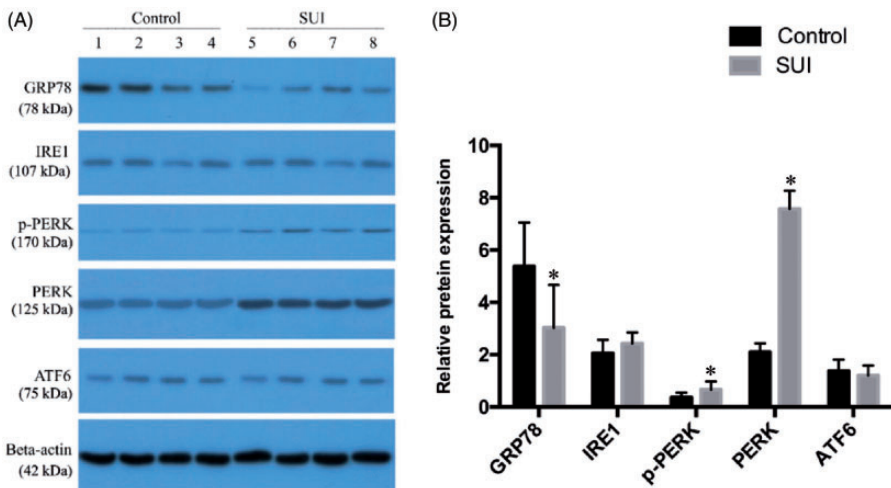

Figure 2. (A) Representative Western blotting for GRP78, IRE1, p-PERK, PERK, and ATF6 proteins in anterior vaginal wall tissue from control subjects (n = 10, lanes 1–4) and women with SUI (n = 10, lanes 5–8). (B) Mean ± standard deviation levels of GRP78, IRE1, PERK, and ATF6, semi-quantified with reference to beta-actin. *$P < 0.05$ vs. control subjects. GRP78, glucose-regulated protein; IRE1, inositol-requiring enzyme 1; PERK, protein kinase-like endoplasmic reticulum kinase; p-PERK, phosphorylated protein kinase-like endoplasmic reticulum kinase; ATF6, activating transcription factor 6; SUI, stress urinary incontinence.
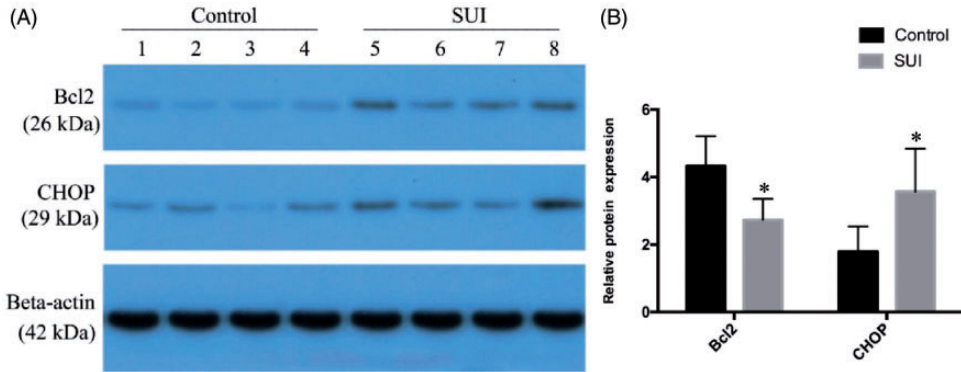
Figure 3. (A) Representative Western blotting for CHOP and Bcl-2 proteins in anterior vaginal wall tissue from control subjects (n = 10, lanes 1–4) and women with SUI (n = 10, lanes 5–8). (B) Mean ± standard deviation levels of CHOP and Bcl-2, semi-quantified with reference to beta-actin.
*$P < 0.05$ vs. control subjects. CHOP, C/EBP-homologous protein; Bcl-2, B-cell lymphoma 2; SUI, stress urinary incontinence.
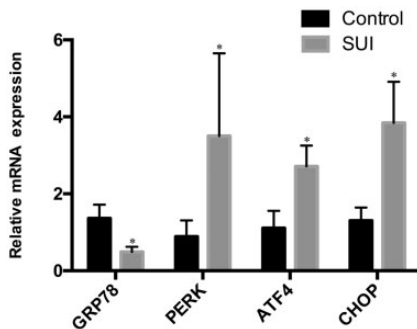

Figure 4. Real-time PCR was performed to measure relative mRNA levels of GRP78, PERK, ATF4 and CHOP in anterior vaginal wall tissue from control subjects (n = 10) and women with SUI (n = 10). Data are expressed as mean ± standard deviation.
*$P < 0.05$ vs. control subjects. PCR, polymerase chain reaction; GRP78, glucose-regulated protein; PERK, protein kinase-like endoplasmic reticulum kinase; ATF4, activating transcription factor 4; CHOP, C/EBP-homologous protein; SUI, stress urinary incontinence.

protein: PERK, IRE1, and ATF6. Our present study showed higher PERK protein expression in postmenopausal women with SUI, suggesting that PERK signaling proteins might play a primary role in UPR

related to the onset of SUI. However, no differences in either IRE1 or ATF6 protein content were observed between groups in this study.

As is well-known, GRP78 acts as a key upstream activator of ERS to restore folding of misfolded or incompletely assembled proteins.[20] Recently, abundant evidence[21,22] has suggested that GRP78 negatively regulates UPR and serves in a protective role for ERS. During ERS, GRP78 dissociates from IRE1, PERK, and ATF6, thus liberating these signaling proteins in order to promote a compensatory defense response.[23] In our study, lower expression of GRP78 was observed in postmenopausal women with SUI, which suggested that the protection of GRP78 may have been significantly weakened during maintenance of ER homeostasis in SUI.

CHOP, also known as GADD153, is a marker of ERS-induced apoptosis. It has been widely described as a transcription factor, which can negatively regulate cell growth and provide robust activation of cell death pathways.[24] Notably, the expression of CHOP is extremely low under non-stress conditions. However, CHOP can be

robustly activated during apoptosis when self-protection mechanisms are overwhelmed.[25,26] In our current study, higher CHOP mRNA and protein expression levels were confirmed in women with SUI; this implied an increased ERS-induced apoptotic effect in these patients. Moreover, Bcl-2 is also a pro-apoptotic protein that is essential in regulating apoptosis. As previously reported,[27,28] a reduction in Bcl-2 protein level is positively associated with upregulation of apoptosis, whereas overexpression of Bcl-2 protein is associated with downregulation of apoptosis. In the present study, the Bcl-2 protein expression level was significantly reduced in women with SUI, suggesting that increased apoptosis may be involved in the development of SUI.

Although the precise mechanisms of ERS-induced apoptosis have not been fully elucidated, excessive or severe ERS may cause high expression of PERK-ATF4-CHOP, thus leading to apoptosis.[29] Typically, when detached from GRP78, PERK mediates auto-phosphorylation and induces a downstream signaling pathway to inhibit protein translation; it then preferentially increases translation of the ATF4 protein, thus restoring ER homeostasis.[30] However, if ERS is excessive in length and/or severity, robustly activated ATF4 protein can stimulate the pro-apoptotic transcription factor CHOP to induce apoptosis by downregulation of Bcl-2 protein levels.[25,31] Our current data demonstrated that the p-PERK and PERK protein levels were increased, and that the PERK, ATF4, and CHOP mRNA levels were significantly upregulated in postmenopausal women with SUI. Supported by the above theory, our data suggest that the PERK-ATF4-CHOP pathway might be associated with the pathogenesis of SUI.

In conclusion, significant alterations of ERS markers in the anterior vaginal wall were demonstrated in postmenopausal women with SUI, suggesting that ERS might be involved in the occurrence of SUI in postmenopausal women. However, the sample size was relatively small; thus, further investigations are needed. We expect that these findings may provide a novel insight into the mechanism of SUI and offer a promising future strategy for the treatment of women with SUI.

Declaration of conflicting interest

Funding

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References

1. Zhu L, Lang J, Liu C, et al. The epidemiological study of women with urinary incontinence and risk factors for stress urinary incontinence in China. *Menopause* 2009; 16: 831–836.

2. Schaffer J, Nager CW, Xiang F, et al. Predictors of success and satisfaction of nonsurgical therapy for stress urinary incontinence. *Obstet Gynecol* 2012; 120: 91–97.

3. Zhu L, Lang JH, Chen J, et al. Morphologic study on levator ani muscle in patients with pelvic organ prolapse and stress urinary incontinence. *Int Urogynecol J Pelvic Floor Dysfunct* 2005; 16: 401–404.

4. Sumino Y, Yoshikawa S, Mori K, et al. IGF-1 as an important endogenous growth factor for recovery from impaired urethral continence function in rats with simulated childbirth injury. *J Urol* 2016; 195: 1927–1935.

5. Chai TC, Richter HE, Moalli P, et al. Inflammatory and tissue remodeling urinary biomarkers before and after mid urethral sling surgery for stress urinary incontinence. *J Urol* 2014; 191: 703–709.

6. Khaminets A, Heinrich T, Mari M, et al. Regulation of endoplasmic reticulum turnover by selective autophagy. *Nature* 2015; 522: 354–358.

7. Iurlaro R and Muñoz-Pinedo C. Cell death induced by endoplasmic reticulum stress. *J FEBS* 2016; 283: 2640–2652.

8. Goldstein DS. Stress, allostatic load, catecholamines, and other neurotransmitters in neurodegenerative diseases. *Cell Mol Neurobiol* 2012; 32: 661–666.

9. Radziszewski P, Borkowski A, Torz C, et al. Distribution of collagen type VII in connective tissues of postmenopausal stress incontinent women. *Gynecol Endocrinol* 2005; 20: 121–126.

10. Abrams P, Cardozo L, Fall M, et al. The standardisation of terminology of lower urinary tract function: report from the standardisation sub-committee of the international continence society. *Neurourol Urodyn* 2002; 21: 167–178.

11. Bump RC, Mattiasson A, Bø K, et al. The standardization of terminology of female pelvic organ prolapse and pelvic floor dysfunction. *Am J Obstet Gynecol* 1996; 175: 10–17.

12. Xie Z, Shi H, Zhou C, et al. Alterations of estrogen receptor-alpha and - beta in the anterior vaginal wall of women with urinary incontinence. *Eur J Obstet Gynecol Reprod Biol* 2007; 134: 254–258.

13. Huang Q, Jin H, Xie Z, et al. The role of the ERK1/2 signalling pathway in the pathogenesis of female stress urinary incontinence. *J Int Med Res* 2013; 41: 1242–1251.

14. Shen XL, Zhang B, Liang R, et al. Central role of Nix in the autophagic response to ochratoxin A. *Food Chem Toxicol* 2013; 69: 202–209.

15. Shen XL, Zhang Y, Xu W, et al. An iTRAQ-based mitoproteomics approach for profiling the nephrotoxicity mechanisms of ochratoxin A in HEK 293 cells. *J Proteomics* 2014; 78: 398–415.

16. Kooptiwut S, Mahawong P, Hanchang W, et al. Estrogen reduces endoplasmic reticulum stress to protect against glucotoxicity induced-pancreatic β-cell death. *J Steroid Biochem Mol Biol* 2014; 139: 25–32.

17. Darling NJ, Balmanno K and Cook SJ. ERK1/2 signalling protects against apoptosis following endoplasmic reticulum stress but cannot provide long-term protection against BAX/BAK-independent cell death. *PLoS One* 2017; 12: e0184907.

18. Kim SR, Kim DI, Kang MR, et al. Endoplasmic reticulum stress influences bronchial asthma pathogenesis by modulating nuclear factor kB activation. *J Allergy Clin Immunol* 2013; 132: 1397–408.

19. Yang J, Chen W, Zhang B, et al. Lon in maintaining mitochondrial and endoplasmic reticulum homeostasis. *Arch Toxicol* 2018; 92: 1913–1923.

20. Liu L, Chowdhury S, Fang X, et al. Attenuation of unfolded protein response and apoptosis by mReg2 induced GRP78 in mouse insulinoma cells. *FEBS Lett* 2014; 588: 2016–2024.

21. Cook KL, Shajahan AN, Wärri A, et al. Glucose-regulated protein 78 controls cross-talk between apoptosis and autophagy to determine antiestrogen responsiveness. *Cancer Res* 2012; 72: 3337–3349.

22. Li J, Ni M, Lee B, et al. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death Differ* 2008; 15: 1460–1471.

23. Zhu G and Lee AS. Role of the unfolded protein response, GRP78 and GRP94 in organ homeostasis. *J Cell Physiol* 2015; 230: 1413–1420.

24. Yang Y, Pei X, Jin Y, et al. The roles of endoplasmic reticulum stress response in female mammalian reproduction. *Cell Tissue Res* 2016; 363: 589–597.

25. Tanaka Y, Ishitsuka Y, Hayasaka M, et al. The exacerbating roles of CCAAT/enhancer-binding protein homologous protein (CHOP) in the development of bleomycin-induced pulmonary fibrosis and the preventive effects of tauroursodeoxycholic acid (TUDCA) against pulmonary

fibrosis in mice. *Pharmacol Res* 2015; 99: 52–62.

26. Morse E, Schroth J, You YH, et al. TRB3 is stimulated in diabetic kidneys, regulated by the ER stress marker CHOP, and is a suppressor of podocyte MCP-1. *Am Physiol Renal Physiol* 2010; 299: F965–F972.

27. Czabotar PE, Lessene G, Strasser A, et al. Control of apoptosis by the Bcl-2 protein family: Implications for physiology and therapy. *Nat Rev Mol Cell Biol* 2014; 15: 49–63.

28. Wu XY, Hao CP, Ling M, et al. Hypoxia-induced apoptosis is blocked by adrenomedullin via upregulation of Bcl-2 in human osteosarcoma cells. *Oncol Rep* 2015; 34: 787–794.

29. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 2012; 13: 89–102.

30. Wang J, Hu X, and Jiang H. ERS-PERK signaling pathway- mediated Nrf2/ARE-HO-1 axis: a novel therapeutic target for attenuating myocardial ischemia and reperfusion injury. *Int J Cardiol* 2016; 203: 779–780.

31. Wu J, Zhao Z, Kumar A, et al. Endoplasmic reticulum stress and disrupted neurogenesis in the brain are associated with cognitive impairment and depressive-like behavior after spinal cord injury. *J Neurotrauma* 2016; 33: 1919–1935.