c-Jun N-terminal kinase/transforming growth factor-β/Smad3 pathway: Is it associated with endoplasmic reticulum stress-mediated renal interstitial fibrosis?

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Abstract. The present study investigated the role of the c-Jun N-terminal kinase (JNK)/transforming growth factor-β (TGF-β)/Smad3 pathway in endoplasmic reticulum stress (ERS)-mediated renal interstitial fibrosis, which would be beneficial for chronic kidney disease (CKD) therapy. In human renal biopsy tissue, the expression levels of glucose-regulated protein 78 (GRP78) and phosphorylated (p)-JNK were examined by immunohistochemical analysis. In renal tubular HK-2 cells, tunicamycin (TM) was used to induce ERS, and the cells were then treated with the chemical ERS inhibitor 4-phenylbutyrate (4-PBA) or the chemical JNK pathway inhibitor SP600125, respectively. Western blotting was then performed in the cells to determine the expression levels of GRP78 and p-JNK proteins, as well as TGF-β/Smad3 pathway-associated proteins, including TGF-β1, p-Smad3, connective tissue growth factor and α-smooth muscle actin. The results revealed that GRP78 and p-JNK were evidently expressed in the renal tissues of patients with CKD, and these expression levels were significantly higher in renal tissues with severe interstitial fibrosis compared with glomerular minor lesion tissues ($P<0.01$ and $P<0.05$, respectively). Furthermore, ERS and JNK pathway inhibition decreased the expression levels of TGF-β/Smad3 pathway signals in cells incubated with TM. ERS pathway inhibition also attenuated the expression levels of p-JNK in HK-2 cells. In conclusion, ERS was observed to serve an important role in the pathogenesis of CKD and may induce renal interstitial fibrosis via the JNK/TGF-β/Smad3 pathway.

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Introduction

The prevalence of chronic kidney disease (CKD) is currently increasing worldwide, making this disease a rising global health concern (1). CKD can result in end-stage renal disease, which is associated with a number of complications, including mineral and bone disorders, anemia, cognitive decline and cardiovascular disease (2). However, no cure exists for CKD at present, and thus the available treatments are primarily aimed at halting or delaying disease progression. Renal interstitial fibrosis serves a key role in the process of progressive renal injury and is a common characteristic of CKD (3). Therefore, inhibition of renal interstitial fibrosis is of great significance in CKD therapy. A great number of studies have reported that excessive endoplasmic reticulum stress (ERS) participated in the development of renal interstitial fibrosis (4-6); however, the exact mechanism is not fully clear.

The endoplasmic reticulum (ER) is an organelle that is responsible for the transmembrane, secretory and ER luminal protein synthesis (7). A disruption in ER proteostasis may occur under tissue fibrosis, leading to an imbalance between the protein folding demand and capacity (8). Glucose-regulated protein 78 (GRP78) is a central regulator of ER homeostasis, and is commonly used as a biomarker for ERS. Under increased ERS, the unfolded protein response triggers the dissociation of GRP78 from three known transmembrane sensors in the ER, including the protein kinase R-like ER kinase, activating transcription factor 6 and inositol-requiring enzyme I (IRE1). Among them, IRE-1 interacts with the TNF receptor-associated factor 2 (TRAF2) and attracts apoptosis signal-regulating kinase-1 (ASK1) to form IRE1-TRAF2-ASK1, which subsequently activates c-Jun N-terminal kinase (JNK) (9,10).

The JNK family of kinases, also known as stress-activated mitogen-activated protein kinases, includes three distinct members, namely: JNK1, JNK2 and JNK3. The JNK1 and JNK2 isoforms are ubiquitously expressed in the majority of tissues, including the kidney, while JNK3 expression is restricted to nervous system tissues (11). Previous studies have suggested that disorder of the JNK signaling pathway serves a pivotal role in several diseases, such as lung fibrosis,

human fibrosarcoma and renal fibrosis (12-14). In addition, it has been reported that JNK was able to mediate the fibronectin and connective tissue growth factor (CTGF) synthesis, suggesting that JNK was involved in tissue fibrosis (12,13). A study conducted by Ma *et al* (14) demonstrated that CC-401, which is a specific JNK inhibitor, suppressed JNK signaling and significantly reduced renal fibrosis in rats with obstructed kidney, indicating that JNK signaling served a pathogenic role in renal fibrosis. It has also been reported that SP600125, another JNK inhibitor, was able to effectively prevent the transforming growth factor (TGF)-β1-induced phosphorylation of Smad3, and the changes in E-cadherin, α-smooth muscle actin (α-SMA) and collagen I expression, which suggested that JNK possibly mediated peritoneal fibrosis through the TGF-β/Smad3 pathway (15). A recent study revealed that 4-phenylbutyrate (4-PBA), a specific inhibitor of ERS, evidently attenuated JNK phosphorylation and TGF-β-induced profibrogenic CTGF, collagen I protein expression in renal tubular NRK-52E cells (16).

Therefore, it can be hypothesized that ESR may mediate renal interstitial fibrosis through the JNK/TGF-β/Smad3 pathway (Fig. 1). In the present study, the expression level of GRP78 was initially examined in human renal tissue. Next, different pathways were inhibited using chemical agents in human tubular HK-2 cells in order to investigate the possible mechanism of ERS-mediated renal interstitial fibrosis.

Materials and methods

Reagents. Tunicamycin (TM; cat. no. T7765) and the 4-PBA (an ERS inhibitor; cat. no. SML0309) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). SP600125 (a JNK inhibitor, cat. no. ab120065) and antibody against phosphorylated (p)-Smad3 (cat. no. ab52903) were obtained from Abcam (Cambridge, UK). The antibody against p-JNK (cat. no. 4668s) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), while antibodies targeting GRP78 (cat. no. 11587-1-AP), TGF-β1 (cat. no. 18978-1-AP), CTGF (cat. no. 23936-1-AP), α-SMA (cat. no. 14395-1-AP) and β-actin (cat. no. 20536-1-AP) were acquired from ProteinTech Group, Inc. (Wuhan, China).

Patients and tissues. A total of 6 patients (3 male and 3 female; age, 29-60 years old) with asymptomatic hematuria, and/or proteinuria (patients without edema, hypertension and/or renal injury but with more than 3 erythrocytes per high-power field by the urine sediment and/or mild proteinuria of less than 1 g/day of protein) or chronic kidney disease (17), were identified in a retrospective review of renal biopsies received at the Suzhou Municipal Hospital (Suzhou, China) from June 2014 to May 2015. Patients with other systemic diseases (including diabetes, heart disease, pulmonary fibrosis, liver fibrosis and neurodegenerative diseases) or disease complications (including infection, anemia and hypoproteinemia) were excluded via examination of clinical history, physical examination and laboratory test results. Paraffin-embedded renal biopsy specimens from these patients were conserved at room temperature. With consent from the patients, paraffin-embedded specimens were sliced into 4 μm sections for immunohistochemical evaluation.

Sample classification and immunohistochemical staining. The six paraffin-embedded specimens, including three specimens of minor glomerular lesion (with asymptomatic hematuria and/or proteinuria, regarded as control group) and three of severe renal interstitial fibrosis (CKD), were evaluated via immunohistochemical staining. According to the World Health Organization (18), glomerular minor lesions are regarded as kidney tissue without evident interstitial fibrosis, while severe renal interstitial fibrosis is defined as kidney tissue with >50% of interstitial fibrosis. The estimated glomerular filtration rates[calculated according to the CKD-EPI formula (19)] of the three patients with glomerular minor lesion were 123.5, 90.1 and 96.7 ml/min, while the respective rates of patients with severe renal interstitial fibrosis were 54.6, 36.7 and 73.6 ml/min.

Paraffin-embedded 4 μm sections were analyzed by immunohistochemical staining. Briefly, the sections were rehydrated, and antigen retrieval was performed with heated citrate. Sections were incubated with 3% H_2O_2 at room temperature for 15 min to remove endogenous peroxidase activity. Immunohistochemical staining was performed using primary antibodies against GRP78 (1:50) and p-JNK (1:20) overnight at 4˚C. Following incubation with a reaction enhancer (OriGene Technologies, Inc., Rockville, MD, USA) at 37˚C for 20 min, sections incubated with horseradish peroxidase labeled goat anti-rabbit immunoglobulin G (IgG) polymer (cat. no. PV9001, OriGene Technologies, Inc.) at 37˚C for 30 min. The signals were developed with DAB Peroxidase Substrate kit (Vector Laboratories, Ltd., Burlingame, CA, USA) at room temperature for 3 min. Brown staining represents the GRP78 and p-JNK expression. Intensity of immunohistochemistry staining was assessed as follows: 0, negative; 1, faint yellow; 2, light brown; and 3, dark brown. The immunohistochemistry staining range was classified as: 1 (0-25% staining of the section); 2 (26-50% staining of the section); 3 (51-75% staining of the section); and 4 (76-100% staining of the section). The final immunohistochemistry staining score was the sum of the intensity scores and staining range scores, and was graded from 1 to 7. All immunohistochemical analyses were repeated at least three times, and representative images are presented.

Cell culture and treatment. All cell experiments were performed using HK-2 cells, a human proximal tubular cell line (supplied by the Second Xiangya Hospital of Central South University, Changsha, China). HK-2 cells were maintained in Dulbecco's modified Eagle's medium/F12 (cat. no. SH30023.01; HyClone; GE Healthcare Life Sciences, Logan, Utah, USA) supplemented with 10% fetal bovine serum (cat. no. P303-3302, PAN-Biotech GmbH, Aidenbach, Germany), 100 μg/ml streptomycin and 100 U/ml penicillin at 37˚C in 5% CO_2. The media were changed every 3 days until confluence was reached. Cells were growth-arrested in serum-free medium for 24 h prior to use in experiments. To determine whether ESR inhibition alleviated the activation of JNK and TGF-β/Smad3 pathway, HK-2 cells were incubated with TM (0.2 μM) for 24 h and then treated with 4-PBA (1.0 mM) for 2 h. Subsequently, to determine whether JNK signaling inhibition alleviated the activation of TGF-β/Smad3 pathway, HK-2 cells were incubated with TM (0.2 μM) for 24 h and then treated with

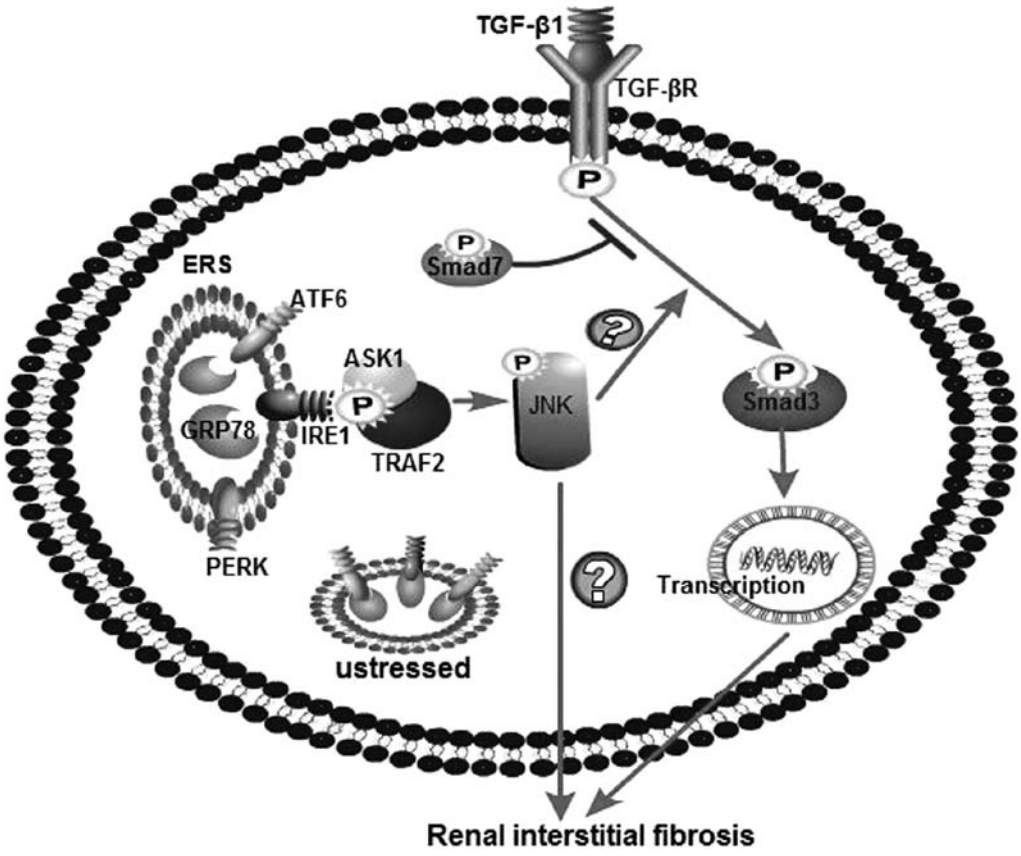

Figure 1. Theoretical basis of the present study. Based on previous studies, it was hypothesized that excessive ESR induced the phosphorylation of JNK through IRE-1-TRAF2-ASK1, upregulated TGF-β/Smad3 pathway signals and finally resulted in renal interstitial fibrosis. JNK, c-Jun N-terminal kinase; TGF-β, transforming growth factor-β; ERS, endoplasmic reticulum stress.

SP600125 (10 μM) for 1 h. The selection of TM, 4-PBA and SP600125 concentrations was based on previous studies and preliminary experiments (20-22).

Western blotting. After treatment, cells were harvested, rinsed with PBS and lysed on ice in RIPA buffer (Applygen Technologies, Inc., Beijing, China) supplied with protease inhibitors (Merck KGaA). The protein concentrations were measured using a bicinchoninic acid assay kit. Equal amount of protein (50 μg total protein) from whole cell lysates was separated by 10% SDS-PAGE and then transferred to nylon membranes. The membranes were blocked in a TBS solution with 5% skimmed milk containing 0.1% Tween-20 at 37˚C for 1 h. Next, the membranes were incubated with primary antibody against GRP78 (1:1,000), p-JNK (1:1,000), TGF-β1 (1:500), p-Smad3 (1:2,000), CTGF (1:500), α-SMA (1:1,000) and β-actin (1:4,000) overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated secondary antibody goat anti-rabbit IgG (cat. no. SA00001-2) or goat anti-mouse IgG (cat. no. SA00001-1; both at 1:3,000; ProteinTech Group, Inc.) at 37˚C for 45 min. Chemiluminescent detection was performed using an enhanced chemiluminescence substrate kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Quantification of the band intensities was conducted using Quantity One version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the intensity was normalized to that of β-actin for standardization.

Statistical analysis. SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for data analysis. The experimental results are expressed as the mean ± standard deviation. Analysis between groups was performed using one-way analysis of variance followed by Tukey post hoc tests for multiple comparisons for normally distributed quantitative data. Categorical data was compared using Mann-Whitney U tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of GRP78 and p-JNK in renal tissues of CKD patients are increased. To investigate the association between ERS and renal interstitial fibrosis, the expression levels of GRP78 and p-JNK were analyzed in human renal tissues of different lesion degrees by immunohistochemical staining. The results revealed evident expression of GRP78 and p-JNK in the renal tissues of CKD patients. In addition, GRP78 and p-JNK expression levels were significantly higher in renal tissues with severe interstitial fibrosis compared with those withglomerularminor lesions ($P<0.01$ and $P<0.05$ for each protein, respectively; Fig. 2), which verified that ERS potentially accelerates the progression of renal interstitial fibrosis.

ESR inhibition alleviates the expression levels of GRP78 and p-JNK in HK-2 cells. To determine whether ESR inhibition

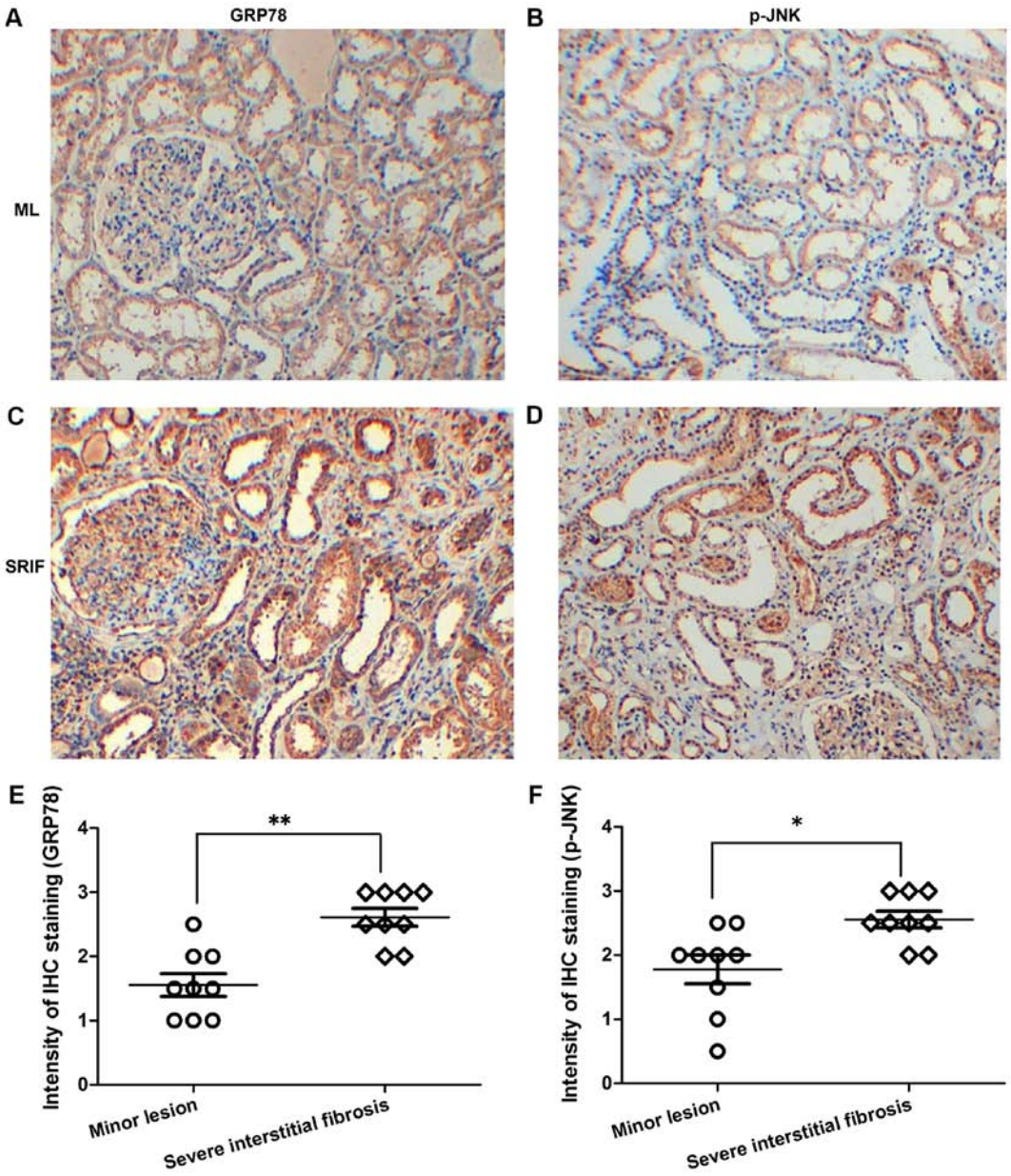

Figure 2. Expression of GRP78 and p-JNK in human renal tissue, examined by IHC staining (magnification, x100). Brown staining represents the GRP78 and p-JNK expression. IHC staining for (A) GRP78 and (B) p-JNK is shown in GML tissues. IHC staining for (C) GRP78 and (D) p-JNK is shown in SRIF tissues. The staining score of (E) GRP78 and (F) p-JNK in SRIF tissues was distinctly increased compared with that in GML tissues. Patients groups were statistically analyzed using a Mann-Whitney U test (n=3 per group). Experiments were repeated three times. *P<0.05 and **P<0.01. GRP78, glucose-regulated protein 78; p-JNK, phosphorylated c-Jun N-terminal kinase; IHC, immunohistochemical; GML, glomerular minor lesion; SRIF, severe renal interstitial fibrosis.

alleviated the activation of JNK, HK-2 cells were incubated with the ESR chemical inducer TM (0.2 μM) for 24 h and then treated with the ESR chemical inhibitor 4-PBA (1.0 mM) for 2 h. The results demonstrated that incubation with TM significantly increased the expression levels of GRP78 and p-JNK proteins compared with the untreated cells (P<0.01 and P<0.001, respectively). However, co-treatment with 4-PBA markedly ameliorated the TM-induced, ERS-associated expression of GRP78 and p-JNK proteins in HK-2 cells (both P<0.01; Fig. 3).

ESR inhibition alleviates the expression of TGF-β/Smad3 pathway-associated proteins in HK-2 cells. To determine whether ESR inhibition alleviated the activation of the TGF-β/Smad3 pathway, HK-2 cells were incubated with the ESR chemical inducer TM (0.2 μM) for 24 h and then treated with the ESR chemical inhibitor 4-PBA (1.0 mM) for 2 h. The results revealed that TM incubation increased the expression levels of TGF-β/Smad3 signals, including TGF-β1, p-Smad3, CTGF and α-SMA protein levels (P<0.01 and P<0.001). By contrast, 4-PBA co-treatment evidently ameliorated the TM-induced expression levels of TGF-β/Smad3 pathway-associated proteins in HK-2 cells (P<0.01 and P<0.001; Fig. 4).

Inhibition of JNK signaling alleviates the expression levels of TGF-β/Smad3 pathway-associated proteins in HK-2 cells. To determine whether JNK signaling inhibition alleviated the

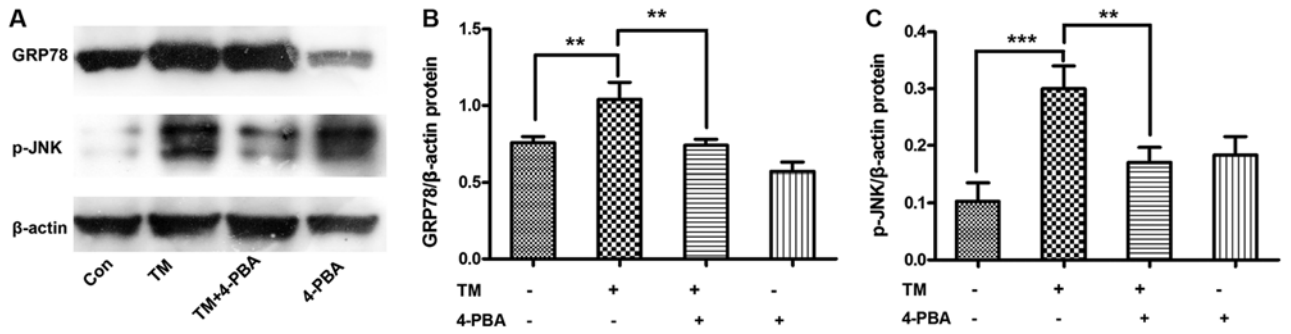

Figure 3. ESR inhibition alleviated the expression levels of GRP78 and p-JNK in HK-2 cells. HK-2 cells were incubated with the ESR chemical inducer TM (0.2 μM) for 24 h and then treated with the ESR chemical inhibitor 4-PBA (1.0 mM) for 2 h. (A) Western blot analysis of cell lysates for GRP78 and p-JNK proteins, with blots reprobed for β-actin. (B) GRP78 and (C) p-JNK protein expression levels relative to β-actin, with pooled data are shown. Data are presented as the mean ± standard deviation, and were assessed by analysis of variance and Tukey post-hoc test (n=3). **P<0.01 and ***P<0.001. ERS, endoplasmic reticulum stress; GRP78, glucose-regulated protein 78; p-JNK, phosphorylated c-Jun N-terminal kinase; TM, tunicamycin; 4-PBA, 4-phenylbutyrate.

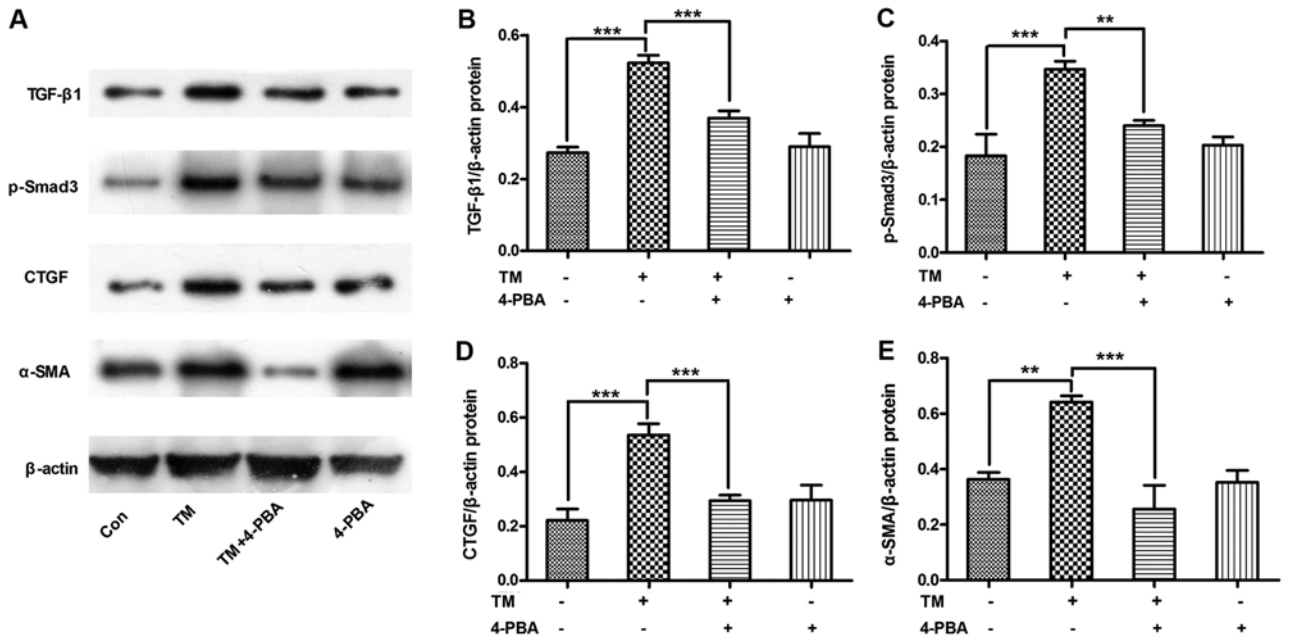

Figure 4. ESR inhibition alleviated the expression of TGF-β/Smad3 pathway proteins in HK-2 cells. HK-2 cells were incubated with the ESR chemical inducer TM (0.2 μM) for 24 h and then treated with the ESR chemical inhibitor 4-PBA (1.0 mM) for 2 h. (A) Western blot analysis of cell lysates for TGF-β1, p-Smad3, CTGF and α-SMA proteins, with blots reprobed for β-actin. (B) TGF-β1, (C) p-Smad3, (D) CTGF and (E) α-SMA protein expression levels relative to β-actin, with pooled data are shown. Data are presented as the mean ± standard deviation, and were assessed by analysis of variance and Tukey post-hoc test (n=3). **P<0.01 and ***P<0.001. ERS, endoplasmic reticulum stress; TM, tunicamycin; 4-PBA, 4-phenylbutyrate; TGF-β1, transforming growth factor-β1; p-Smad3, phosphorylated Smad3; CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin.

activation of TGF-β/Smad3 pathway, HK-2 cells were incubated with the ESR chemical inducer TM (0.2 μM) for 24 h and then treated with the JNK pathway chemical inhibitor SP600125 (10 μM) for 1 h. The data indicated that SP600125 treatment significantly decreased the TM-induced p-JNK expression (P<0.01) and the expression levels of TGF-β/Smad3-associated proteins, including TGF-β1 (P<0.01), p-Smad3 (P<0.05), CTGF (P<0.01) and α-SMA (P<0.01), in HK-2 cells (Fig. 5).

Discussion

Renal tubulointerstitial fibrosis is the common and final pathologic change of the kidney in end-stage renal disease (23). Inhibition of renal interstitial fibrosis is of great significance in CKD therapy. Previous cell and animal experiments have suggested that ERS was implicated in the development of renal interstitial fibrosis (4,16). In the present study, immunohistochemical staining revealed that GRP78 was evidently expressed in the renal tissues of CKD patients. In addition, higher GRP78 expression was detected in renal tissue with severe interstitial fibrosis as compared with that of minor glomerular lesion tissues, indicating the potentially key role of ERS in the renal interstitial fibrosis process, consistent with the observations of previous studies (4,16).

TGF-β is regarded as a central mediator of renal interstitial fibrosis (3,24,25), and its upregulation occurs in nearly all types of CKD. TGF-β acts on downstream signaling through Smad phosphorylation. Smad proteins are highly conserved transcription factors that are central to signal transduction pathways and mediate numerous effects associated with the

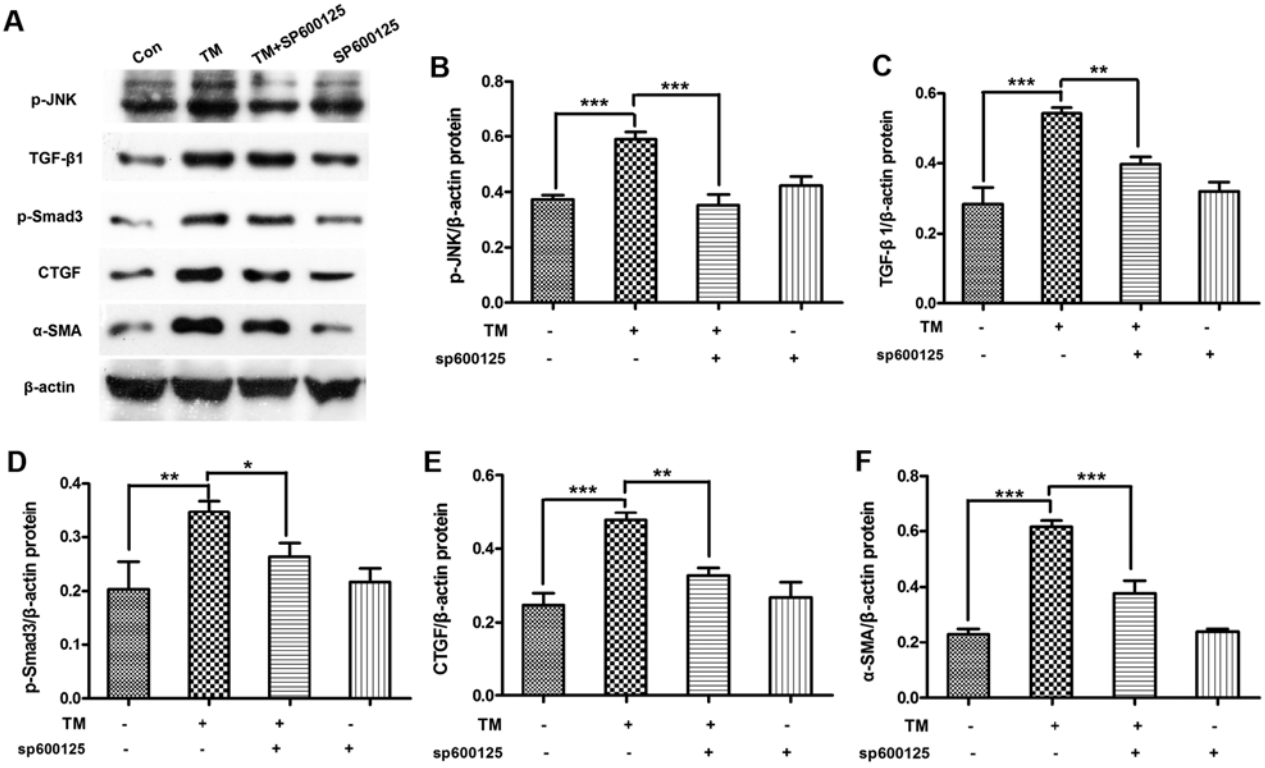

Figure 5. Inhibition of JNK signal alleviated TGF-β/Smad3 pathway expression in HK-2 cells. HK-2 cells were incubated with the ESR chemical inducer TM (0.2 μM) for 24 h and then treated with the JNK pathway chemical inhibitor SP600125 (10 μM) for 1 h. (A) Western blot analysis of cell lysates for p-JNK, TGF-β1, p-Smad3, CTGF and α-SMA, with blots reprobed for β-actin. (B) p-JNK, (C) TGF-β1, (D) p-Smad3, (E) CTGF and (F) α-SMA protein expression graphs relative to β-actin, with pooled data are shown. Data are presented as the mean ± standard deviation, and were assessed by analysis of variance and Tukey post-hoc test (n=3). *P<0.05, **P<0.01 and ***P<0.001. JNK, c-Jun N-terminal kinase; TGF-β, transforming growth factor-β; ERS, endoplasmic reticulum stress; TM, tunicamycin; p-, phosphorylated; CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin.

TGF-β superfamily signaling pathway (26). Among them, Smad3 is a key mediator in renal fibrosis, whereas Smad2 and Smad7 exhibit renal-protective properties (27,28). In addition, CTGF is considered to be determinant of progressive renal fibrosis and a downstream mediator of TGF-β1 signaling in the fibrosis process (29,30). During the fibrosis process, epithelial-mesenchymal transition, characterized by the loss of cell adhesion markers and the *de novo* expression of mesenchymal markers such as α-SMA, is considered to be an indicator of interstitial fibrosis (31). In the present study, TM was used to activate ERS in HK-2 cells, which resulted in increased expression levels of TGF-β1, p-Smad3, CTGF and α-SMA. In contrast, treatment with 4-PBA to inhibit ERS led to decreased expression levels of these proteins, which further indicated that ERS may have served a key role in the development of renal interstitial fibrosis.

Currently, it remains unclear how ERS induces renal interstitial fibrosis. Previous research has shown that ERS mediated tubular cell apoptosis that then resulted in renal interstitial fibrosis (4,16). Additionally, a number of other studies revealed that JNK participates in the development of tissue fibrosis, including in lung, liver and ovarian tissues, among others (13,32,33). Based on previous animal experiments, Ma *et al* (14) reported that JNK signaling served a pathogenic role in renal interstitial fibrosis. Consistent with previous studies, the data of the current study revealed that p-JNK was evidently expressed in human renal tissues obtained from CKD patients, and its expression level was correlated with the interstitial fibrosis degree, further suggesting the potential role of JNK signaling in renal interstitial fibrosis.

Understanding the mediation of renal interstitial fibrosis by JNK, a downstream signaling molecule of the ERS, is also of great significance. A study by Liu *et al* (15) reported that blocking the activation of JNK with SP600125 resulted in the ineffective inhibition of TGF-β1-induced phosphorylation of Smad3, suppressed the TGF-β1-induced upregulation of α-SMA and collagen I, and prevented the TGF-β1-induced downregulation of E-cadherin expression in rat peritoneal mesothelial cells. These observations suggested that JNK possibly mediated peritoneal fibrosis through the TGF-β/Smad3 pathway (15). According to the present study, the expression levels of p-JNK, TGF-β1, p-Smad3, CTGF and α-SMA in HK-2 cells were increased when ERS was activated by TM. However, their expression levels were decreased when the cells were subsequently treated with 4-PBA, which suggested that ERS potentially activated TGF-β/Smad3 signaling through JNK pathway regulation, which may have resulted in renal interstitial fibrosis. In addition, JNK blocking with SP600125 resulted in downregulation of TGF-β1, p-Smad3, CTGF and α-SMA levels in HK-2 cells, which further verified the aforementioned hypothesis.

A potential limitation of the present study is the absence of normalization of phosphorylated proteins to the total proteins. Nevertheless, the upregulation of p-JNK is indicative of the potential role of the JNK pathway in ERS-induced renal interstitial fibrosis.

In conclusion, the present study demonstrated that ERS possibly mediated renal interstitial fibrosis through the JNK/TGF-β/Smad3 pathway, which may provide a useful insight for CKD therapy. However, due to limitations in the methods of the current study, the exact mechanism of ERS-mediated renal interstitial fibrosis remains to be further clarified by *in vivo* studies through genetic methods, such as transfection with small interfering RNA or gene knockout.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HC and YL were responsible for the experimental design. YL and PS performed the majority of the experiments and drafted the manuscript. YZ and LT participated in sample classification and immunohistochemical staining. YL and LT assisted with analyzing the experimental results. HC, YZ and PS revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The described experiments were approved by the Scientific and Ethics Committee of the Affiliated Suzhou Hospital of Nanjing Medical University (Suzhou, China; permit no. KL901006) prior to conducting the study. All patients provided written informed consent prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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