

# Ameliorating Effect of Klotho on Endoplasmic Reticulum Stress and Renal Fibrosis Induced by Unilateral Ureteral Obstruction

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**Introduction.** Expression of Klotho is decreased and endoplasmic reticulum (ER) stress is activated in patients with chronic kidney disease. This study aimed to investigate the effect of Klotho protein on ER stress-related apoptosis and renal fibrosis in rats with unilateral ureteral obstruction (UUO).

**Materials and Methods.** Twenty-four rats were divided into the sham, UUO, and Klotho treatment groups. Renal interstitial fibrosis model was induced by UUO in the rats of the latter two groups. Soluble Klotho protein was injected into the abdominal cavity. Serum and kidney samples were collected 14 days after the UUO surgery for examination of renal injury and renal interstitial fibrosis using hematoxylin-eosin and Masson trichrome staining methods. The level of apoptotic cells was assessed by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Expression of 3 ER stress-related proteins including glucose-regulated protein 78, CCAAT/enhancer-binding protein homologous protein, and caspase-12 were measured.

**Results.** Kidney dysfunction, increased renal injury index, and aggravated renal fibrosis were documented in the UUO group. Expression of Klotho in the UUO rats was remarkably decreased ( $P < .05$ ) and expression of all three ER stress-related proteins were significantly upregulated, accompanied by increasing numbers of apoptotic cells ( $P < .05$ ). Soluble Klotho administration improved kidney function, ameliorated pathological changes, decreased expressions of ER-stress related-proteins, and reduced the number of apoptotic cells.

**Conclusion.** Unilateral ureteral obstruction decreased Klotho expression and activated ER stress and related apoptosis. Klotho administration ameliorated UUO-induced ER stress, inhibited apoptotic process, and attenuated renal fibrosis.

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## INTRODUCTION

Klotho protein is encoded by the *KL* gene and loss of Klotho leads to multiple age-associated disorders, suggesting Klotho functions as a novel anti-aging protein.<sup>1,2</sup> Also, studies have shown

aging is related to the development of CKD,<sup>3</sup> and Klotho protein, with its anti-aging property, is implicated in the pathogenesis of kidney diseases. Since Klotho is expressed mainly in renal tissues,<sup>4</sup> it is not surprising that Klotho is decreased in the

diseased kidneys.<sup>5,6</sup> Deficiency of Klotho leads to cell apoptosis in kidneys and overexpression of *KL* results in inhibition of cell apoptosis.<sup>7,8</sup> Furthermore, Klotho protein treatment ameliorates apoptosis and fibrosis in the kidneys affected by ischemia-reperfusion and unilateral ureteral obstruction (UUO), suggesting that the protective mechanism may be ascribed to inhibition of cell apoptosis.<sup>2,9,10</sup>

The endoplasmic reticulum (ER) is an important organelle and exerts important functions in the regulation of protein synthesis and processing. In addition, the ER also plays a critical role in controlling the death or survival of cells and serves as a significant inducer of cell apoptosis.<sup>11</sup> Insults from oxidative stress and hypoxia can disturb the function of ER and lead to increased unfolded or misfolded proteins, which were accumulated in the ER lumen. In order to maintain ER function, ER stress is triggered. Nevertheless, ER stress-induced apoptosis ensues if ER stress is unable to alleviate the stressors.<sup>12</sup> Recent studies show that ER stress-induced apoptosis is closely associated with fibrogenic processes,<sup>13</sup> including kidney fibrosis,<sup>14</sup> yet the relationship between Klotho and ER stress in renal fibrosis has not been explored. In this study, we induced renal fibrosis in a rat model via UUO and investigated the effect of Klotho on ER stress-mediated apoptosis and renal fibrosis.

## MATERIALS AND METHODS

Twenty-four Sprague-Dawley male rats, which were purchased from Shanghai Laboratory Animal Research Center, were divided randomly into 3 groups ( $n = 8$ , each group): sham group, UUO group, and Klotho treatment group. The UUO rat model was developed as described previously.<sup>15</sup> The dosage of recombinant rat Klotho protein (Life Sciences, USA) was 0.02 mg/kg, given by intraperitoneal injection in Klotho treatment group. The dosage was determined according to the literature.<sup>10</sup> An equivalent volume of physiological saline was given to the sham group and UUO group. The same treatments were given by every other day until the rats were sacrificed 14 days after the UUO surgery. All animal experiments were approved by the local Animal Care and Use Committee.

Blood samples were collected and blood urea nitrogen (BUN) and serum creatinine were measured with an auto-analyzer (Roche Modular P, USA).

A part of the obstructed kidney was fixed in 10% formalin for subsequent standard paraffin sections. Renal sections at 4- $\mu$ m thickness were stained with hematoxylin-eosin and Masson trichrome based on the procedure. The parameters of renal interstitial injury, including inflammatory cell infiltration, interstitial edema, tubular atrophy, interstitial fibrosis, and tubular expansion were measured to evaluate the severity of the renal lesions. Each parameter was assessed and graded on a scale from zero to 4 (zero, no changes; 1, changes affecting < 25%; 2, changes affecting 25% to 50%; 3, changes affecting 50% to 75%; 4, changes affecting 75% to 100% of the section).<sup>16</sup> The severity of interstitial fibrosis was estimated by scanning 10 no-repeated fields in each sample with Masson trichrome staining. Blue-stained fibrotic areas were quantified by the Image-Pro plus 6.0 software (Media Cybernetics, Rockville, MD, USA).<sup>17</sup> The results were expressed by the proportion of the relative volume of the scanned interstitium.

Renal tubular cells undergoing apoptosis were identified in the 4- $\mu$ m paraffin-embedded sections using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in situ apoptosis kit (QIA33, Merk, USA) according to the manufacturer's protocol. For apoptotic tubular cells counting, TUNEL-positive cells were counted by 2 observers in a blinded manner in 10 randomly selected fields per slide ( $\times 400$  magnification).

Kidney samples frozen in liquid nitrogen were lysed with ice-cold radioimmuno-precipitation assay buffer. Protein extraction and immunoblot analysis were performed as previously described.<sup>18</sup> Primary antibodies used in this study were Klotho (SAB3500604, Sigma-Aldrich), glucose-regulated protein 78 (GRP78; ab21685, Abcam), CCAAT/enhancer-binding protein homologous protein (CHOP; #2895, Cell Signaling Technology), caspase-12 (PRS2325, Sigma-Aldrich), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; A106610, GenScript). Secondary antibodies used in this study were rat immunoglobulin G-horseradish peroxidase (Solarbio, Beijing, China), mouse immunoglobulin G-horseradish peroxidase (ZB-2305, ZSGB Bio, Beijing, China), and goat immunoglobulin G-horseradish peroxidase (Solarbio, Beijing, China). Signals were detected using the Bio-Rad system (Bio-Rad, Hercules,

CA, USA), and the densitometric analysis were performed using the Quantity One software (Bio-Rad, Hercules, CA, USA). Expression of GAPDH was used as the internal standard.

Statistical analysis was performed using the SPSS software (Statistical Package for the Social Sciences, version 22.0, SPSS Inc, Chicago, Ill, USA). All quantitative results were expressed as mean  $\pm$  standard deviations. Differences in the mean values between the rat groups were analyzed using the 1-way analysis of variance test, followed by the least-significant-difference test as a post-hoc test. *P* values less than .05 were considered to significant.

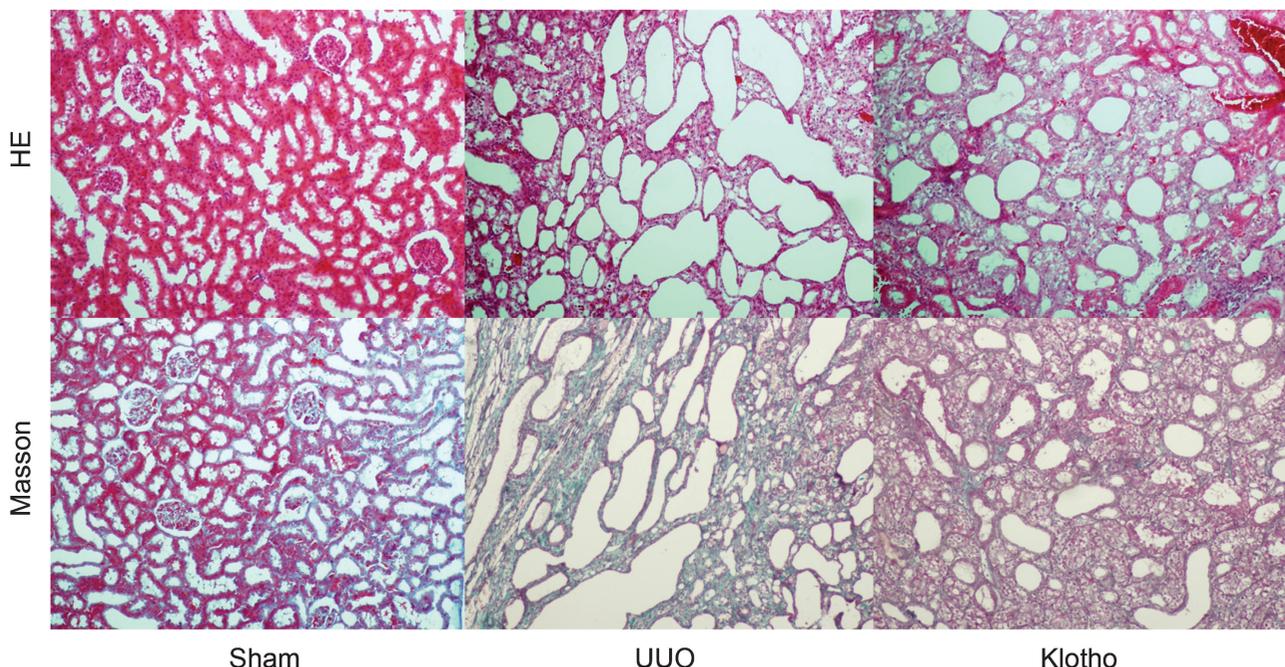
## RESULTS

Kidney function was aggravated by UUO, represented as the increasing levels of serum creatinine ( $75.00 \pm 6.08$   $\mu\text{mol/L}$  versus  $32.00 \pm 3.61$   $\mu\text{mol/L}$ , *P* < .001) and BUN ( $12.47 \pm 2.60$  mg/dL versus  $5.93 \pm 0.61$  mg/dL, *P* = .003). Klotho administration improved kidney function (serum creatinine,  $51.67 \pm 9.50$   $\mu\text{mol/L}$  versus  $75.00 \pm 6.08$   $\mu\text{mol/L}$ , *P* = .006; BUN,  $8.40 \pm 1.11$  mg/dL versus  $12.47 \pm 2.60$  mg/dL, *P* = .03).

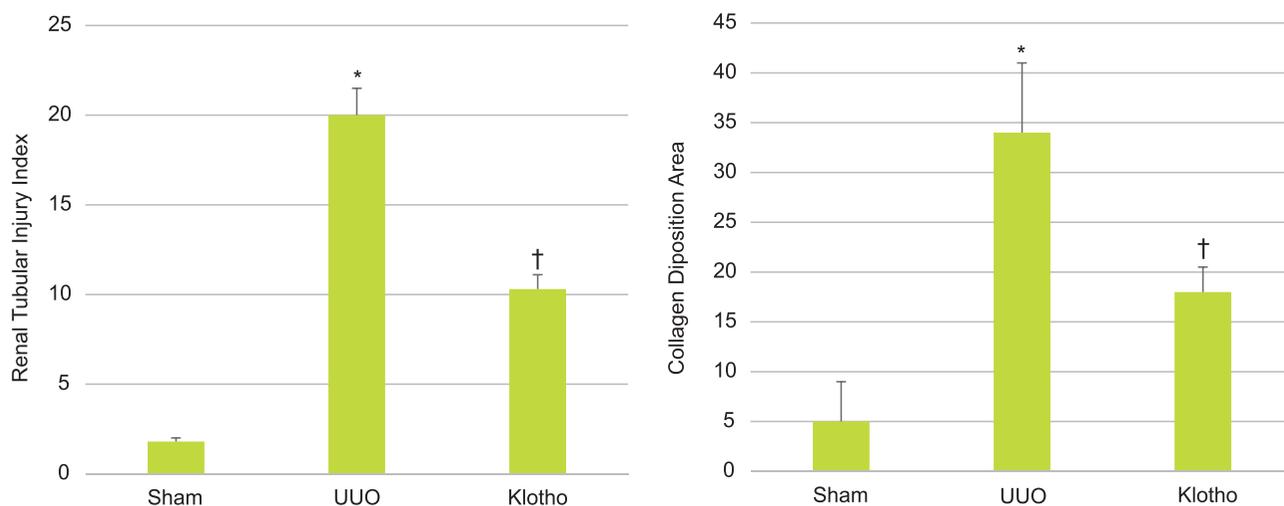
There were no histological lesions in the sham group by hematoxylin-eosin staining. Fourteen days after UUO, the kidneys developed remarkable

pathological changes such as interstitial fibrosis, tubular expansion, and atrophy and inflammatory cell invasion (Figure 1). Consistent with the pathological changes in the experimental kidneys, UUO surgery resulted in a 11-time increase of the renal tubular injury index, but the extent of damage was remarkably blunted (0.52 times that of the UUO group) by the administration of soluble Klotho (Figure 2). Masson trichrome staining showed that collagen accumulation had prominently increased about 6 folds in the interstitium in the UUO kidneys, while administration of soluble Klotho decreased the amount of collagen deposition from  $34.75 \pm 2.21\%$  to  $18.50 \pm 0.82\%$ , compared to the UUO group (*P* < .001; Figures 1 and 2). Unilateral ureteral obstruction increased the number of TUNEL-positive cells in the cortex from  $0.8 \pm 0.83$  per high-power field (HPF) to  $22.60 \pm 6.88$  per HPF (*P* < .001), whereas administration of Klotho decreased the number of TUNEL-positive cells to  $15.40 \pm 3.05$  per HPF (*P* = .02; Figure 3).

Compared with the sham group, expression of Klotho in kidneys decreased significantly in the UUO group ( $0.45 \pm 0.04$  versus  $1.65 \pm 0.07$ , *P* = .005), but Klotho treatment increased its expression ( $1.38 \pm 0.33$  versus  $0.45 \pm 0.04$ , *P* = .02; Figure 4A). As shown in Figure 4B, GRP78 was induced remarkably after UUO ( $1.38 \pm 0.13$  versus



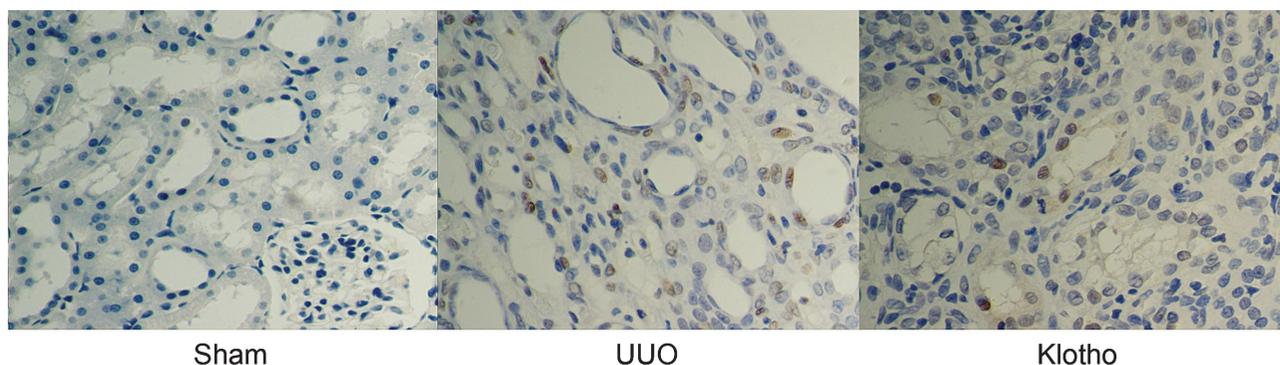
**Figure 1.** Klotho minimized unilateral ureteral obstruction (UUO)-triggered pathological changes in the kidneys. **Top**, Hematoxylin-eosin (HE) staining ( $\times 200$ ). **Bottom**, Masson trichrome staining ( $\times 200$ ).



**Figure 2.** **Left,** The semi-quantitative accession of the renal interstitial lesions in hematoxylin-eosin-stained sections of rat kidneys of the sham, unilateral ureteral obstruction (UUO), and Klotho groups. **Right,** The degree of interstitial collagen deposits in Masson trichrome-stained sections.

\**P* < .01 compared to the sham group

†*P* < .01 compared to the UUO group



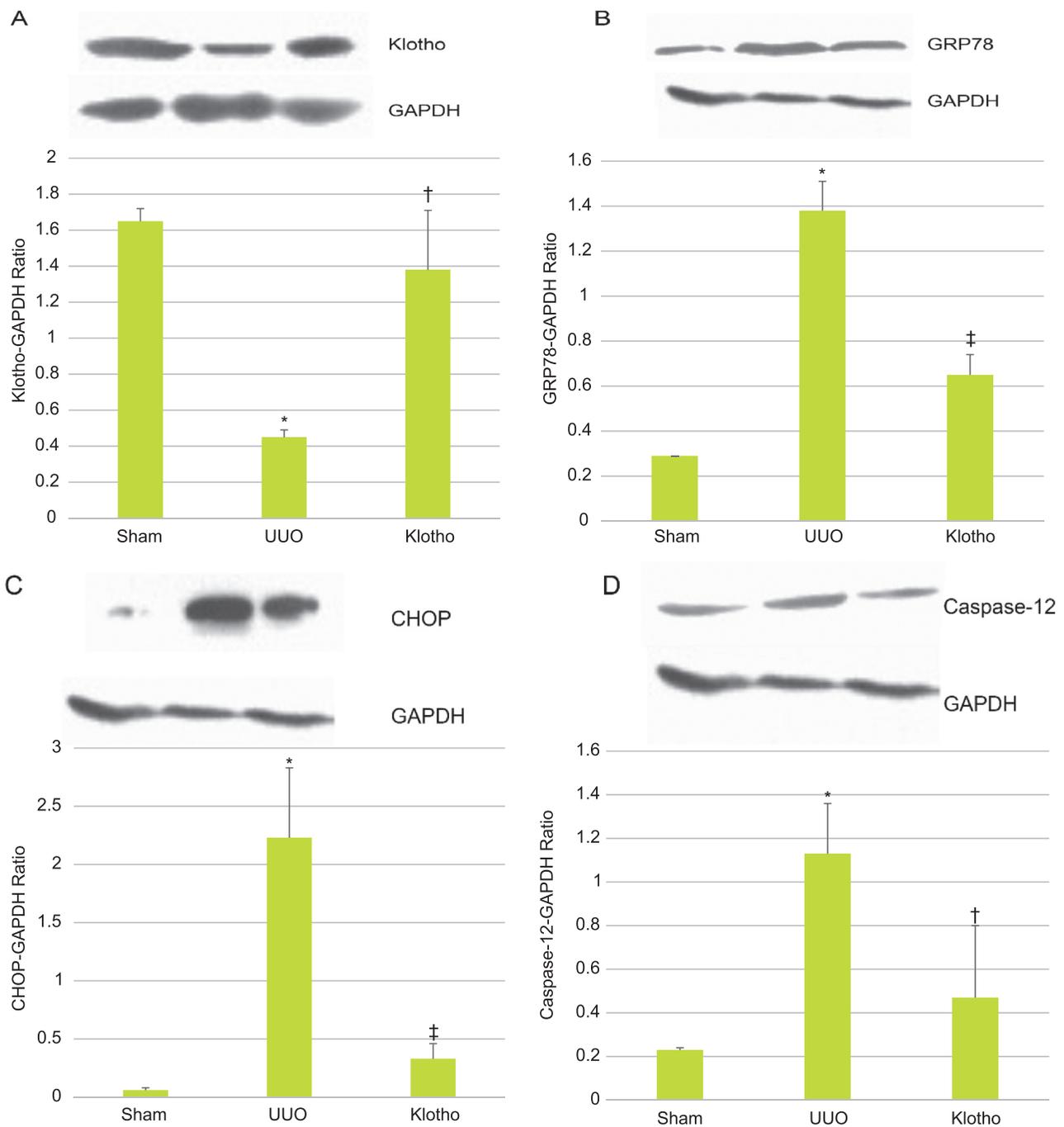
**Figure 3.** Cells apoptosis observed using the terminal deoxynucleotidyl transferase dUTP nick end labeling staining of rat kidneys of the sham, unilateral ureteral obstruction (UUO), and Klotho groups ( $\times 400$ ).

$0.29 \pm 0.0$ , *P* < .001). Expression of GRP78 in the Klotho treatment group was significantly lower than that of the UUO group ( $0.65 \pm 0.09$  versus  $1.38 \pm 0.13$ , *P* = .002).

As shown in Figure 4C, CHOP protein was dramatically elevated in the UUO group compared with the sham group ( $2.23 \pm 0.60$  versus  $0.06 \pm 0.02$ , *P* = .005), but soluble Klotho administration substantially ameliorated this elevation induced by UUO surgery ( $0.33 \pm 0.13$  versus  $2.23 \pm 0.60$ , *P* = .009). Similar results were obtained in caspase-12 expression among the experimental groups (Figure 4D). The expression of caspase-12 was increased by about 5 folds compared to that in the sham group ( $1.13 \pm 0.23$  versus  $0.23 \pm 0.01$ , *P* = .003), while the administration of Klotho alleviated its increase ( $0.47 \pm 0.33$  versus  $1.13 \pm 0.23$ , *P* = .013).

## DISCUSSION

Amounting studies have revealed Klotho is able to suppress cell apoptosis and inhibits renal fibrosis by interfering pro-fibrotic growth factors and signalings.<sup>1,10-12,19</sup> These indicate that Klotho may be a modifier of cell apoptosis and fibrosis in kidneys. It is known that cell apoptosis can result in inflammatory response, fibroblast phenotypic switch, and stimulating synthesis of collagen directly or indirectly.<sup>20</sup> Apoptosis enhances fibrotic remodeling process in internal organs,<sup>21,22</sup> and apoptosis in the renal tubular cells leads to tubular atrophy followed by renal fibrosis.<sup>23</sup> Three apoptotic pathways are involved in cell apoptosis, including ER pathway, mitochondrial pathway, and death receptor pathway. Increasing evidence reveals that apoptosis induced by ER pathway



**Figure 4.** Effect of Klotho on the expression of CCAAT/enhancer-binding protein homologous protein (CHOP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-regulated protein 78 (GRP78), and caspase-12 proteins in renal tissue of rat kidneys of the sham, unilateral ureteral obstruction (UUO), and Klotho groups.

\* $P < .01$  compared to the sham group

† $P < .05$  compared to the UUO group

‡ $P < .01$  compared to the UUO group

plays an important role in the progression of kidney diseases.<sup>15,16</sup>

Prolonged ER stress fails to restore homeostasis and triggers apoptotic pathway. Overproduction of reactive oxygen species and hypoxia due to UUO

lead to unfolded proteins accumulated in the ER and subsequent release of the ER chaperone GRP78, which results in activation of ER stress and unfolded protein response.<sup>24,25</sup> Endoplasmic reticulum stress and unfolded protein response serve to restore

the ER homeostasis by enhancing capacity of protein folding and accelerating the degradation of the unfolded proteins. If the stressors are not removed or remain persistent, unfolded protein response-mediated apoptosis occurs via CHOP and caspase-12 pathways.<sup>26</sup> The CHOP induced by persistent ER stress stressors, is an important regulator protein linking ER stress to apoptosis.<sup>27</sup> Caspases are required for cell apoptosis as well. Unlike other caspases, caspase-12 is exclusively expressed in the ER and is absolutely activated by ER stress-induced factors, not other apoptotic stimuli.<sup>26</sup> Caspase-12 and CHOP are considered as indicators of apoptosis mediated by ER stress.

Two recent reports have elaborated that loss of Klotho aggravated ER stress-induced apoptosis in human renal proximal tubular epithelial cells in vitro and cardiomyocyte cells in vivo,<sup>28,29</sup> and firstly illustrated the role for Klotho in inhibiting ER stress-induced apoptosis. Whether Klotho may mitigate cell apoptosis by decreasing ER stress in UUO-induced fibrotic kidneys has not been reported. In order to clarify the relationship of Klotho with ER stress-related apoptosis, we developed a model of renal fibrosis in rats through UUO surgery. Our results showed that UUO led to deterioration of kidney function after 14 days. Unilateral ureteral obstruction also aggravates renal tubular atrophy, renal interstitial fibrosis, and inflammation and cell infiltration. Western Blot analysis showed that expression of Klotho in the kidney in UUO was significantly downregulated ( $P < .001$ ), indicating that UUO significantly decreased Klotho expression. Whereas, the administration of soluble Klotho protein improved kidney function, relieved pathological changes and reduced renal damage index (all  $P < .05$ ). This shows Klotho functions as a protective protein and Klotho deficiency may implicate in the pathogenesis of renal fibrosis in UUO.

In order to elucidate the mechanism that Klotho inhibits apoptosis, we evaluated the effect of Klotho on ER stress-related apoptosis. We measured GRP78, CHOP, and caspase-12 to illustrate the mechanism underlying the apoptotic process. The TUNEL analysis was conducted to estimate the degree of cell apoptosis. We found that UUO increased expressions of ER stress-related proteins, including GRP78, CHOP, and caspase-12. In addition, the number of apoptotic cells observed

by the TUNEL staining significantly increased in UUO rats. However, administration of soluble Klotho protein decreased significantly the levels of GRP78, CHOP, and caspase-12 and markedly reduced the number of apoptotic cells. These data demonstrated that UUO led to ER stress-related cell apoptosis, and Klotho treatment inhibited ER stress-induced apoptosis. The mechanism by which Klotho inhibited apoptosis is possibly attributed to the amelioration of ER stress in the UUO rats.

## CONCLUSIONS

Our study revealed that Klotho plays an important role in mitigating ER stress and related apoptosis in UUO rat model. Soluble Klotho protein can alleviate ER stress and related apoptosis and finally reduce renal interstitial fibrosis. Klotho protein, with its antiapoptotic and antifibrotic properties, may serve as a potential therapeutic agent for replacement therapy in Klotho-deficient states. But the relevant mechanism is still to be further studied.

## CONFLICT OF INTEREST

None declared.

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