Early Elimination of Uremic Toxin Ameliorates AKI to CKD Transition

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ABSTRACT

Acute kidney injury (AKI)-related fibrosis is emerging as a major driver of chronic kidney disease (CKD) development. Aberrant kidney recovery after AKI is multifactorial and still poorly understood. The accumulation of indoxyl sulfate (IS), a protein-bound uremic toxin, has been identified as a detrimental factor of renal fibrosis. However, the mechanisms underlying IS-related aberrant kidney recovery after AKI is still unknown. The present study aims to elucidate the effects of IS on tubular damage and its involvement in the pathogenesis of AKI to CKD transition. Our results showed that serum IS started to accumulate associated with the downregulation of tubular organic anion transporter, but not observed in the small-molecule uremic toxins of the unilateral ischemia-reperfusion injury (UIRI) without a contralateral nephrectomy model. Serum IS is positively correlated with renal fibrosis and Binding immunoglobulin protein (BiP) and CAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) expression induction in the UIRI with a contralateral nephrectomy model (UIRI+Nx). To evaluate the effects of IS in the AKI to CKD transition, we administered indole, a precursor of IS, at the early stage of UIRI. Our results demonstrated IS potentiates renal fibrosis, senescence-associated secretory phenotype (SASP), and activation of endoplasmic reticulum (ER) stress, which is attenuated by synergistic AST-120 administration. Furthermore, we clearly demonstrated that IS exposure potentiated hypoxia-reperfusion (H/R) induced G2/M cell cycle arrest, epithelial-mesenchymal transition (EMT), and aggravated ER stress induction in vitro. Finally, the ER chemical chaperon, 4-phenylbutyric acid (4-PBA), successfully reversed the above-mentioned AKI to CKD transition. Taken together, early IS elimination in the...
early stage of AKI is likely to be a useful strategy in the prevention and/or treatment of the AKI to CKD transition.

**Key words:** AKI to CKD; indoxyl sulfate; AST-120; ER stress; unfolded protein response

**CLINICAL PERSPECTIVES**

It has been well known that uremic toxins removal attenuated the renal fibrosis in the advanced CKD both *in vitro* and *in vivo*. However, it is still unclear whether early removal of IS, a representative protein-bound uremic toxin, will attenuate the following kidney progression in the AKI stage.

We found for the first time that IS sustained accumulation without BUN and serum creatinine increasing in UIRI model and early IS elimination by AST-120 retards AKI to CKD transition through attenuation of SASP and ER stress.

Future studies should now examine the therapeutic potential of AST-120 and 4-PBA (a chemical ER chaperon) in AKI patients and its influences on AKI-to-CKD transition.
INTRODUCTION

Acute kidney injury (AKI) is characterized by a rapid decline of renal function [1], which associates with the increasing risks of subsequential comorbidity, including acute myocardial infarction, heart failure, and sepsis. Recently, it has been identified that AKI is a long-term risk factor for chronic kidney disease (CKD), end-stage renal disease (ESRD), and death [2]. Some patients can fully recover from AKI, but others cannot because maladaptive repair after AKI leads to chronic kidney fibrosis [3]. The rising burden of CKD after AKI has pushed the study to focus on the early treatment of AKI and enhance adequate renal function recovery. However, even the normal serum creatinine (Scr) level after AKI does not always truly reflect the complete recovery from AKI [4].

With renal function deterioration, the accumulation of various endogenous metabolites is generally named uremic toxins [5]. Indoxyl sulfate (IS), a protein-bound uremic toxin, is the product of indole sulfation in the liver via the portal circulation after uptaken metabolite of tryptophan by the gut bacteria [6]. Because of its high protein bond characteristics, IS is secreted through the organic anion transporter (OTA) of renal tubular epithelial cells (RTECs) and systemically accumulates in CKD patients [7]. Recently, it has caught more attention for its systemically detrimental effects in CKD patients [6, 8, 9], but not in the AKI population.

It is well documented that the unfolded protein responses (UPRs) signaling is activated by the derangement of endoplasmic reticulum (ER) proteostasis and participates in various kidney injuries [10]. Our previous study demonstrated the ER stress implicated in the development of renal fibrosis [11]. Furthermore, abundant pieces of evidence reported a high association between ER dysfunction and premature...
aging [10], and CKD is increasingly being accepted as a type of renal aging. During AKI to CKD transition, senescence-associated secretory phenotype (SASP), which is associated with the secretion of interleukin-6 (IL-6), tumor necrotic factor-α (TNF-α), and plasminogen activator inhibitor 1 (PAI-1), actively contributed to the progression of CKD [12, 13]. This study hypothesized that the early uremic toxin removal in AKI would modify the harmful effects through modulation of UPRs and SASP and retarded AKI to CKD transition. To clarify this question, we use the unilateral ischemia-reperfusion with/without contralateral nephrectomy model to explore the early removal of IS by AST-120, an oral spherical carbonaceous adsorbent, on AKI to CKD progression in vivo. Furthermore, we also investigated the molecular mechanisms of IS exposure to H/R conditions in vitro. Our results support that the early removal of IS attenuates renal fibrosis after ischemic AKI.

MATERIALS AND METHODS

Animal models and protocol

C57BL/6 mice aged from 8 to 10 weeks were used in this study. All procedures in this study were approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine (IACUC NO. 20170164) and all the surgeries were conducted at the operation room in National Taiwan University College of Medicine Laboratory Animal Center. The AKI to CKD transition mouse model: Unilateral ischemic-reperfusion injury without contralateral nephrectomy (UIRI) and two-stage unilateral ischemic-reperfusion injury model with contralateral nephrectomy (UIRI+Nx) at day10 were introduced in this experiment [14, 15]. For both UIRI models, mice were anesthetized with intraperitoneal injection of Zoletil (50
mg/kg)/ Xylazine (2 mg/kg) mixture, and the body temperature was maintained at 37.5°C during surgery with a temperature-controlled operating table (Physitemp Instruments, Clifton, New Jersey). At the beginning of stage I (day 0), UIRI was induced by unilateral clamping of the left renal pedicle with a hemoclip for 30 min. The right kidney was kept intact. The wound was sutured after the release of the clip, and animals were allowed to recover. At the beginning of stage II (day 10), the right kidneys were removed, and animals were sacrificed to observe CKD transition at indicated time points or the end of observation on day 15. The next day after nephrectomy was expressed as NxD1, and the observative endpoint was named as UIRI+Nx. All mice in each group are divided into indole (IS precursor, 10µg/g/day via gavage) or AST-120 (4mg/g/day via oral feeding) treatment at the 5th day of two-stage UIRI, and were allocated into 5 groups, including (1) Sham, (2) UIRI, (3) UIRI + indole, (4) UIRI + indole+ AST-120, (5) UIRI + AST-120 groups. At the end of the experimental procedure, all the animals were anesthetized and euthanized by cardiac puncture then cervical dislocation. The animal serum and kidney samples were collected for further analysis.

Reagents and antibodies

IS and sodium phenylbutyrate were purchased from Sigma-Aldrich. Indole and anaerobic bags were purchased from Merck KGaA (Darmstadt, Germany). KREMEZIN (AST-120) was purchased from Conmed Pharmaceutical & Bio-Medical Corporation. The following antibodies were used: Phospho-eIF2α (Ser51, 9721S), eIF2α (9722S), and CHOP (2895S) were purchased from Cell Signaling Technology (Danvers, MA); SLC22A6 (ab135924), E-Cadherin (GTX124178), Vimentin (GTX100619), α-SMA (GTX100034), β-actin (GTX109639), GAPDH (GTX100118), anti-rabbit IgG (H + L), and anti-mouse IgG (H + L) antibodies were
purchased from Genetex (Hsinchu City, Taiwan). Cyclin B1 (sc-245), Cyclin D1 (sc-246), p-Smad2/3 (sc-11769-R), Smad2/3 (sc-6032), and CTGF (sc-14939) were purchased from Santa Cruz Biotechnology (Dallas, TX). BiP (610978) was purchased from BD Biosciences.

**Serum indoxyl sulfate measurement**

Blood samples were collected by cardiac or submandibular puncturing and allowed samples to clot at room temperatures for 30 minutes. The sample was centrifuged at 4 °C 1500 × g for 15 min for serum collection. Serum levels of IS were measured by liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS, perform by National Taiwan University Hospital, Forensic and Clinical Toxicology Center).

**Cell culture**

The HK-2, a human proximal tubular epithelial cell line, was obtained from Bioresource Collection and Research Center (Hsinchu City, Taiwan) and was cultured in DMEM/F12 (1:1) medium (ThermoFisher Scientific, Waltham, MA) supplemented with 10 % fetal bovine serum (Biological Industries) at 37 °C in a 5% CO2-humidified environment. To generate the IS exposure and ischemia-reperfusion environment in vitro, we applied the Anaerocult A mini system (Merk, Whitehouse Station, NJ), which consumes the oxygen content to 0.2% to mimic hypoxic conditions. For the experimental procedure, HK-2 cells were treated with 1mM IS for 24 hours, then undergoing H/R insult. After 12 h of anoxia, HK-2 cells were moved to the normoxic incubation chamber after being replaced fresh medium for 24 hours. For 4-PBA rescued experiment, HK-2 cells were treated with or without 1mM 4-PBA after H/R.
**Immunoblotting**

Cells or kidney tissue were lysed in RIPA buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). The lysate was centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatants were collected, and the protein concentrations were measured by the Coomassie Protein Assay Reagent (ThermoFisher Scientific). Equal amounts of proteins were (30-40 µg) boiled with 4x protein sample buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, and 5% beta-mercaptoethanol) for 10 minutes then separated by 8-15% SDS-PAGE gel and electrotransferred to the polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with TBST (0.2% Tween 20 (vol/vol)) containing 5% nonfat milk for 1 hour, then incubated with primary antibodies at 4ºC overnight. Horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated at room temperature for 1h and developed with Immobilon Western HRP Chemiluminescent Substrate (Millipore). The chemiluminescent image was captured with the BioSpectrum 810 Imaging System (UVP, Upland, CA).

**RNA extraction, reverse transcription, RT-PCR, and quantitative real-time PCR**

Total RNA of cells or freshly isolated kidneys were extracted by GENEzol™ TriRNA Pure Kit (Geneaid, New Taipei City, Taiwan). One microgram of RNA was reverse transcribed with iScript reverse transcription supermix (Bio-Rad, Hercules, CA). The resulting cDNA products were amplified with specific primer pairs to detect mRNA abundance using a StepOnePlus real-time PCR system (ThermoFisher Scientific). The relative expression of the target genes was calculated using the comparative threshold cycle (CT) method (ΔΔCT). The sequences of the primer pairs are listed in Table 1.
**Intracellular Reactive Oxygen Species detection**

DCFDA / H2DCFDA - Cellular Reactive Oxygen Species (ROS) Detection Assay Kit was purchased from abcam (ab113851, Cambridge, UK). Cultured HK-2 cells were treated with IS and 12 h hypoxia, then incubated with 25 μM DCFDA for 45 min at 37°C. The fluorescence was excited by the 488 nm laser and detected at 535 nm using Paradigm Multi-Mode Plate Reader (Beckman Coulter PARADIGM).

**Cell cycle analysis**

After washing twice with ice-cold phosphate-buffered saline (PBS), cells were collected and fixed using 70% ethanol at 4°C for at least 30 minutes. Then the cells were washed twice with ice-cold PBS and stained with 50 μg/ml propidium iodide (P4170, Sigma, Continental, USA) in the presence of 5 μg RNase A (Thermo Fisher Scientific, New Hampshire, USA) at room temperature for 30 minutes prior to analysis using flow cytometry (BD LSRII) with excitation laser 561 nm and bandpass filter 670/30. Data was output as FACS files and analyzed by FlowJo software.

**Histological analysis**

Kidneys were removed from euthanized mice and fixed in 10% formalin with phosphate-buffered saline (PBS). Hematoxylin and eosin-stained sections were used to estimate renal histological injury. Masson’s trichrome-stained sections were used to evaluate renal fibrosis which is measured by imageJ blue-stained area to cross the whole image represented as fibrosis area fraction for the degree of interstitial collagen deposition. Twenty cortical tubulointerstitial fields that were randomly selected at 400X magnification were assessed in each mouse, and the average for each group was analyzed.
**Immunohistochemistry staining**

Fresh kidney samples were fixed with 10% formalin, embedded in paraffin, and then sectioned into 4-μm sections. Tissue sections were deparaffinized using xylene and rehydrated with 100%, 95%, 85%, and 70% ethanol. For antigen unmasking, slides in sodium citrate buffer were boiled for 15 min. Slides were blocked with 1% bovine serum albumin for 1 h and then immersed in PBS containing antibodies for organic anion transporter 1 (OAT 1:250; ab135924) overnight. After washing with PBS, slides were immersed in PBS containing a biotin-conjugated secondary antibody for 1 hour. After washing with PBS, samples were incubated in streptavidin-horseradish peroxidase solution. Finally, 3,3’ diaminobenzidine was used as a chromogenic substrate, forming brown deposits on a reaction with horseradish peroxidase.

**MTS assay**

CellTiter 96® Aqueous One Solution Cell Proliferation kit (Promega, Wisconsin, USA) was used in MTS assay. [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt] (MTS) assay is a colorimetric method for sensitive quantification of cell proliferation. In living cells, the reduction of MTS tetrazolium compound by NAD(P)H-dependent dehydrogenase enzymes generates a colored
formazan product. HK-2 cells were plated in a 96 well plate. After washing each
treatment cells with PBS, a fresh culture medium containing 0.2% MTS was added to
each well and incubated at 37°C for 45 minutes. The absorbance was measured at 490
nm and 650 nm on the SpectraMax® ABS Plus Microplate plate reader. Data were
normalized to controls and represented as the proliferation rate of the controls.

Statistical Analysis

All data examined are expressed as means ± SEMs. Statistical analyses of the data
were performed using Prism 6 software. Comparison between groups was made using
one-way ANOVA followed by Newman–Kuels test. P < 0.05 was considered
statistically significant.
RESULTS

Accumulation of serum IS in UIRI without contralateral nephrectomy model

As shown in Figure 1A, the UIRI protocol demonstrated the time-point of observation through the study period. The tissue and blood samples were collected for histopathology and biochemistry analysis at indicated days post-UIRI. As shown in Figure 1B, the H&E staining showed an apparent tubular dilation, the hallmark feature of AKI, at day 11 post-UIRI (UD11). Small molecular uremic toxins including, blood urea nitrogen (BUN), and Scr were not raised, but IS was significantly accumulated in UIRI mice compared with that of sham-operated mice (Figure 1C and 1D). Because IS was secreted by organic anion transporter, we further demonstrated renal OAT-1 expression significantly decreased in UIRI kidney tissue (Figure 1E). In addition, BUN and Scr levels were significantly accumulated at NxD1 mice (Figure 1F and 1G). These findings suggested that serum IS accumulated since the early stage of UIRI while impairing organic acid secretion of the injured kidney, but BUN and Scr accumulated only after impaired glomerular filtration developed while the non-ischemic kidney is removed.

Serum IS accumulation was associated with post-AKI fibrosis

To monitor the fibrosis progression after UIRI, we stained kidney tissue sections with Masson’s Trichrome (MT), which represented the extracellular matrix deposits [16].
As shown in Figures 2A and 2B, in the UIRI group, the MT staining area gradually increased, and more than 20% collagen deposition was formed from UD11 to UD15. Concomitantly, the protein expression of α-SMA and vimentin were also substantially increased in UIRI mice kidneys (Figure 2C-E), and similar was also found in Col1α1 mRNA expression (Figure 2F). We hypothesize that IS plays as a trigger for fibrosis development, then further evaluate the relationship between serum IS level and fibrotic/epithelial-mesenchymal transition (EMT) parameters. As shown in Figure 2G and 2H, serum IS level was significantly positive correlated with α-SMA (Pearson’s r = 0.7436, P < 0.001), and also positively correlated with fibrosis fraction (Pearson’s r = 0.6561, P < 0.05). These results suggest that IS may be a possible detrimental factor in the transition from AKI to CKD in the UIRI kidney.

**AST-120 administration attenuated renal fibrosis in two-stage UIRI with contralateral nephrectomy**

In order to demonstrate the early removal of protein-bound uremic toxin attenuated the renal fibrosis in the two-stage UIRI mice, we randomly assigned into indole (10 µg/g/day via gavage) or AST-120 (4 mg/g/day via oral feeding) treatment 5 days after UIRI, including (1) sham; (2) UIRI+Nx; (3) UIRI+Nx+indole; (4) UIRI+Nx+indole+AST-120; and (5) UIRI+Nx+AST-120 groups as shown in Figure 3A. First, the UIRI+Nx group showed significantly serum IS accumulation, further
increasing after indole administration (Figure 3B). Furthermore, tubulointerstitial damage and fibrosis were also demonstrated by H&E and Masson’s trichrome staining (MT) in Figure 3C. These results suggested that high-protein intake through indole supplement might enhance kidney injury during AKI to CKD transition. As shown in Figures 3B and 3C, AST-120 treatment effectively reduced the serum IS accumulation, which concomitantly abolished the tubulointerstitial damage and fibrosis. Further study revealed that transforming growth factor-beta 1 (TGF-β1) and connective tissue growth factor (CTGF), which are the critical mediators in promoting renal fibrosis, and extracellular matrix gene col1a1 mRNA expression in the indole gavage group were significantly upregulated (Figure 3D-F) and decreased in IS removal group (indole+AST-120 and AST-120 treatment group). Moreover, EMT plays a critical role in AKI to CKD transition, and we further analyzed the effects of AST-120 on CTGF, vimentin, and α-SMA protein expression. As shown in Figure 3G-3J, AST-120 significantly attenuated CTGF, vimentin, and α-SMA expression in UIRI+Nx+indole+AST-120 and UIRI+Nx+AST-120 groups, and the downstream target of TGF-β1: p-Smad2/3 protein expression had similar trends in IS accumulation and elimination group (Figure 3G and 3K). These results suggested the therapeutic potential of early IS removal by AST-120 in the AKI to CKD transition.
AST-120 treatment modulated UPRs and SASP in AKI to CKD transition

Our previous work and others study demonstrated a significant association between kidney disease progression and proteostasis imbalance [10]. Therefore, we further observed UPR signaling of the study groups. As shown in Figure 4A-4C, BiP and CHOP protein expression were upregulated in the UIRI+Nx group and further enhanced in the UIRI+Nx+indole group; AST-120 treatment partially suppressed their expression. The expression of CHOP has been previously implicated in renal pathological processes in vitro and in vivo [9]. We further demonstrated the serum IS levels positively associated with BiP and CHOP protein (Figure 4D-E), which indicated the IS might contribute to the induction of UPRs. Furthermore, we examined the expression of SASP in the two-stage UIRI kidney. As compared with the sham group, the UIRI+Nx group significantly increased the mRNA expression of *IL-6*, *TNF-α*, and *PAI-1* (Figure 4F-H), and indole feeding further upregulated the expression of these factors in AKI mice. AST-120 administration showed significantly reduced *IL-6* and *PAI-1*, but not *TNF-α*, between the indole-treated mice with and without AST-120 administration. These results suggested that oral AST-120 ameliorated AKI to CKD transition, at least partially, through the modulation of SASP.
The mechanisms of IS administration in hypoxia-reperfusion injury *in vitro*

In order to elucidate the roles of IS accumulation in AKI to CKD transition, we further investigated the mechanisms through exposure of IS in hypoxia-reperfusion (H/R) condition by treating with IS (1mM) for 48 hours, then placed into the H/R condition (12 hours hypoxia and 24 hours reperfusion) in HK2 cell. First of all, due to the proliferation inhibition effect of IS has been found in several studies [9, 17, 18], MTS assay was performed to monitor cell proliferation rate. As shown in Supplementary Figure 1, both IS and IS with H/R groups showed a significant cell growth inhibition. Furthermore, cell cycle distribution was also measured in our experiment. We clearly demonstrated that cell cycle G2/M phase arrest was significantly induced in the H/R condition pre-treated with the IS group. Cyclin B1 to cyclin D1 protein ratio, which implicated cell cycle arrest [19], was also significantly upregulated in the H/R condition treated with the IS group (Figure 5C). These results supported that IS potentiated maladaptive repairing processes through prolonged cell cycle arrest. Current evidence suggested that ROS attacked DNA, which forming γ-H2AX, is an early cellular response to the induction of DNA double-strand breaks, resulting in cell cycle arrest [20]. As shown in Figures 5D and 5E, pretreatment of IS aggravated ROS generation, accompanied by γ-H2Ax protein induction in H/R injury. In maladaptive RTECs, cell cycle G2/M phase arrest would enhance EMT and
profibrotic factor upregulation [21]. In Figure 5 F-I, the H/R condition pre-treated with IS had higher profibrotic factor CTGF, α-SMA protein expression, and lower E-cadherin expression level in HK2 cells. In addition, UPRs were also involved in the pretreatment of IS in the H/R condition, as shown that BiP, p-eIF2α, and CHOP protein expression were significantly upregulated (Figure 5J-M). These findings showed that IS potentiated EMT and UPRs in H/R tubular cells, and we further designed the study to clarify the effects of UPRs modulation on the profibrotic phenotype.

4-PBA, an ER chemical chaperon, reversed IS-potentiated cell cycle arrest and EMT in vitro

In order to clarify the roles of IS on the ER proteostasis on the two-stage UIRI kidney (Figure 4A), 4-PBA was treated after H/R. As shown in Figures 6A and 6B, 4-PBA significantly attenuated eIF2α phosphorylation and BiP protein expression. In addition, 4-PBA treatment also restored E-cadherin expression and decreased α-SMA expression levels, which indicated the feature of EMT were attenuated by 4-PBA treatment. (Figure 6C and 6D). Furthermore, IS-potentiated G2/M arrest was also abolished by 4-PBA (Figure 6E and 6F). These results supported ER stress directly contributed to IS-enhanced cell cycle arrest and EMT in H/R injury.
DISCUSSION

AKI is encountered in one-third of in-hospital patients [22]. Current diagnostic criteria of AKI are generally dependent on the kidneys’ filtration function, such as urine output and Scr, but do not include the secretory function of the kidney [23]. Current evidence suggests that IS, a representative protein-bound uremic toxin, mainly excretes by tubular secretion and acts as a pathologic factor that triggers the ROS generation, organelle stress, apoptosis, and inflammation leading to cardiovascular and kidney dysfunction [6, 24, 25]. However, there is a lack of evidence about the IS on AKI to CKD transition. In this study, we found that early removal of IS by AST-120 during AKI successfully attenuated the transition of AKI-to-CKD fibrosis through inhibition of ROS, ER stress, SASP, and profibrotic factors in vivo. Furthermore, IS potentiated ROS generation, DNA damage, cell cycle arrest, ER stress, and EMT, which 4-PBA abolished in vitro. Therefore, our results support that early removal of IS in AKI further restores the adaptive ability and maintains the tubular function, which probably serves as a novel therapeutic approach to AKI patients. There are several ways in which accumulated serum IS may potentiate AKI to CKD injury.

First, small molecular uremic toxins, such as BUN and Scr, are freely filtered and accumulated until severely impaired filtration function. In contrast, endogenous
protein-bound uremic toxins, such as IS, are mainly excreted by anionic transporter localized specifically at the basolateral and brush-border membranes of the proximal tubular cells. The previous study has shown that the downregulation of renal OAT1 and OAT3, which is responsible for increased serum IS level of the injured kidney [21]. In order to demonstrate the different accumulation of uremic toxins between small molecular and protein-bound uremic toxins in AKI, we conduct UIRI without nephrectomy, which maintains adequate glomerular filtration function in the normal kidney and impaired excretory function in the injured kidney. Figures 1C and 1D show that BUN and Scr levels remain within normal limits, but the IS level accumulates as the OAT1 expression is downregulated (Figure 1E, 1F, and 1G). These results support that persistent IS accumulation in AKI even after glomerular filtration function being restored. To elucidate the potentially harmful effects of IS in AKI, we evaluate the correlation between serum IS level and tissue fibrosis. Our results show that a higher serum IS level is positively associated with higher \( \alpha \)-SMA expression and fibrosis staining in the injured kidney (Figure 2G and 2H). These results further support the adverse effects of IS in renal fibrosis during AKI to CKD transition.

Second, previous studies have shown that higher dietary protein food will also be underestimated by using Scr as a biomarker in AKI to CKD transition [26, 27]. IS is
a notorious uremic solute, generally accumulated in CKD patients with deteriorated renal function, impaired neovascularization, and aggravated uremic sarcopenia [8, 28].

A previous study has found that the metabolic profiles change and IS accumulates in the early stage of AKI [29]. The present study further demonstrates that IS is significantly accumulated in the UIRI+Nx group and further boosted by oral indole gavage in the UIRI+Nx+Indole group, as shown in Figure 3B. Recently, an increasing number of articles explored the functional roles of microbiota [30]; IS-producing microflora has aroused more attention in CKD patients [31, 32]. Thus, a low-protein diet has been recommended to non-dialysis CKD patients to reduce the kidney burden from tryptophan-derived uremic toxins [33]. Applying AST-120 successfully attenuated the fibrotic area (Figure 3C) and expression of profibrotic factors, TGFβ1 signaling, and EMT in AKI to CKD tissue (Figure 3D-3K). However, there is a point to mention that AST-120 administration would absorb IS precursor and other PBUT precursors such as p-cresol in the intestine. To emphasize the specific deteriorated effect of IS in AKI to CKD, we designed the analysis into the UIRI+Nx+Indole group and UIRI+Nx+Indole+AST-120 group to clear up this concern. Overall, our findings further support the early elimination of IS attenuates the AKI to CKD insult.

Third, our previous studies and others clearly demonstrate that activation of ER stress is critical for tubular cell loss and renal fibrosis in the AKI to CKD models.
This finding connects a possible causative role for ER stress with the development of progressive kidney fibrosis [10]. In this study, we also evaluate the key indicators of ER stress, including BiP and CHOP, in the UIRI+Nx model. As shown in Figure 4A-C, BiP and CHOP protein have been upregulated in UIRI+Nx and UIRI+Nx+Indole group, which is attenuated by AST-120 administration in trend. In addition, serum IS level is positively correlated with the expression of BiP and CHOP. A similar observation has shown that IS-treated HK2 cells potentiate the H/R-induced BiP, CHOP, and eIF2α phosphorylation (Figure 5J-5M). Previous studies also confirmed that the chemical chaperon 4-PBA protects against ER stress-mediated renal fibrosis [34, 35]. This study, we successfully demonstrate 4-PBA to abolish the IS-induced ER stress in the H/R injury model in vitro (Figure 6A and 6B). According to the above findings, removal of IS in the early stage of AKI might be beneficial for AKI to CKD injury through blunting ER stress-mediated kidney injury both in vivo and in vitro. In addition, age-related organ dysfunction and tissue fibrosis have suggested that chronically senescent cells with a shared SASP are involved in tissue inflammation and fibrosis [22]. A great number of articles indicate AKI as a condition causing premature kidney senescence [36-38]. Adijiang A et al. demonstrates that IS reduces Klotho expression and promotes senescence in the kidney through ROS production, nuclear factor kappa B activation, and enhanced the SASP in proximal
tubular cells [39]. In this study, early IS removal by AST-120 successfully suppresses the IL-6 and PAI-1 expression as shown in the UIRI+Nx+indole+AST-120 group (Figure 4F and 4G). Therefore, removing IS, a well-known proinflammatory activator, would take into concern in the early stage of AKI patients to prevent the development of kidney senescence.

Finally, in response to renal insults surviving renal tubular cells can activate an intrinsic repair process by reentering mitosis and restoring kidney architecture and function. However, the renal repair is limited; even if the renal function returns to baseline after an acute insult, residual inflammatory and fibrotic processes in the kidney can contribute to CKD development [3]. A recent study has linked epithelial cell cycle arrest in G2/M to kidney fibrosis after injury. It is highly associated with proinflammatory and profibrotic cytokines secretion [19]. Our study demonstrates that IS and H/R induce HK-2 cells in G2/M cell cycle arrest and increase cyclin B1/D1 ratio, and IS further potentiates the effects (Figure 5A, 5B, and 5C). The phenomenon is probably associated with ROS generation and following DNA damages by IS exposure in the H/R injured RTECS (Figure 5D and 5E). Furthermore, 4-PBA also attenuates the IS-induced cell cycle arrest in the H/R injury model in vitro (Figure 6E and 6F).
CONCLUSION

Findings from the present study are presented schematically in Figure 7. The findings strongly support the early elimination of IS in AKI. It will further restore the cellular adaptive function by blocking the IS-mediated ROS generation, ER stress induction, SASP, EMT, and profibrotic factors. The ER chaperon 4-PBA also restores IS-induced HK2 cell cycle arrest and E-cadherin suppression. It is warranted to conduct a clinical study by early eliminating IS or modulating ER stress-related maladaptation to uncover the unmet medical needs in AKI patients.
ACKNOWLEDGEMENT

We thank the Second Core Laboratory of the Department of Medical Research in the National Taiwan University Hospital for equipment and facility support.

COMPETING INTERESTS

The authors declare no competing financial interests.

FUNDING

This work was supported by the Ministry of Science and Technology of Taiwan [grant numbers MOST-104-2314-B-002-126-MY3, 107-2314-B-002-027-MY3 and 110-2314-B-002-130]; the National Taiwan University [NTUH-106-S3574, NTUH-107-S3826, and NTUH-110-S5063]; Taipei Veterans General Hospital-National Taiwan University Hospital Joint Research Program [TVGH-NTUH-VN107-18].

AUTHOR CONTRIBUTION

JHC conducted major experiments and data analysis. JHC, and CKC contributed to the design and the writing of the manuscript. CTC, JWH, KYH, DCT and SHL provided reagents and critical comments.
DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of the present study are available from the corresponding author upon reasonable request. The full uncropped and unedited versions of the Western blot images are listed in the supplementary figure.

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Table 1. Information of real-time PCR primers used in this study

| Name   | Species | Forward | Reverse |
|--------|---------|---------|---------|
| Col1α  | Mouse   | CTCCTCTTAagggCCACT | CCACgTCTCAACCATTgggg |
| CTGF   | Mouse   | gTggAATATTgCCggTgCA | CCATTgAAgCATCTTggTTCg |
| PAI-1  | Mouse   | CACAggCACTgCAAAAAggTC | TgTgCCgAACCACAAGAgAgA |
| IL-6   | Mouse   | ACCgCTATgAAgTTCCTCTC | CCTCTgTgAAgTCTCCTCTC |
| TNF-α  | Mouse   | CCACCACgCTCTTCTgTCTAC | AgggTCTgggCCATAgAAGCT |
| TGF-β1 | Mouse   | TgACgTCACTggAgTTgTACgg | ggTTCATgTCATggATggTgC |
| Abbreviation | Meaning |
|--------------|---------|
| 4-PBA        | 4-phenylbutyric acid |
| AKI          | Acute kidney injury |
| AST-120      | KREMEZIN |
| BiP          | Binding immunoglobulin protein |
| BUN          | Blood urea nitrogen |
| CHOP         | CAAT/enhancer-binding protein (C/EBP) homologous protein |
| CKD          | Chronic kidney disease |
| eIF2α        | Eukaryotic translation initiation factor 2α |
| EMT          | Epithelial-mesenchymal transition |
| ER           | Endoplasmic reticulum |
| ESRD         | End-stage renal stage |
| H/R          | Hypoxia-reperfusion |
| HRP          | Horseradish peroxidase |
| Nx           | Nephrectomy |
| OAT          | Organic anion transporter |
| PBS          | Phosphate-buffered saline |
| SASP         | Senescence-associated secretory phenotype |
| Scr          | Serum creatinine |
| TIF          | Tubulointerstitial fibrosis |
| IS           | Indoxyl sulfate |
| RTEC         | Renal tubular epithelial cell |
| ROS          | Reactive oxygen species |
| UIRI         | Unilateral ischemia-reperfusion injury |
| UPR          | Unfolded protein response |
FIGURE LEGENDS

Figure 1. Indoxyl sulfate accumulation without the synergetic small molecules uremic toxin increasing in UIRI mice.

(A) Illustration of experimental design for UIRI. C57BL/6 mice were subjected to 30 minutes of unilateral renal ischemia-reperfusion surgery (UIRI). UDx: x days after UIRI. (B) H&E staining represents the tubular dilation after mice were subjected to UIRI. Scale bar indicates 100μm in 100x, 20 μm in 400x. (C) Scr and BUN levels after UIRI. Scr and BUN levels do not significantly change due to the compensatory effect of the normal contralateral kidney. (D) Serum indoxyl sulfate levels significantly increase after UIRI. (n= 6 for each group). Data are represented as mean ± S.E.M. (E-G) Organic anion transporter 1 expression decreases after UIRI. Result presented by immunohistochemistry staining and western blot analysis. Scale bar indicates 50μm. (H, I) BUN and Scr levels were significantly elevated after contralateral renal nephrectomy. NxD1: one day after nephrectomy.

Figure 2. Correlation between serum IS levels and fibrosis fraction or α-SMA protein expression.

(A) Renal fibrosis represented by Masson’s trichrome stain. Scale bar indicates 50μm.

(B) The renal fibrosis section was quantified and presented in the fibrosis area fraction. (C-E) Protein expression levels of α-SMA and vimentin in kidneys over time.
after subjecting to UIRI were evaluated by western blotting. (F) qPCR assessment of the relative expression level of *Col1a1* mRNA in mice UIRI kidney. Data are represented as mean ± S.E.M. N= 3 for each group. * p< 0.05, ** p< 0.01, *** p < 0.001, as compared with sham.  (G, H) Correlation efficiency of serum IS-fibrosis fraction and serum IS-α-SMA level represented as Pearson’s r.

**Figure 3. The therapeutic effects of uremic toxins removal during AKI to CKD progression.**

(A) Illustration of experimental design for the therapeutic effects of AST-120 intakes during AKI to CKD progression animal model. (B) Serum IS levels in each treatment group. (C) The effects of AST-120 treatment on uremic toxin-induced renal pathology changes after IRI. Scale bar = 20μm. (D-F) qPCR assessment of the relative expression level of *TGF-β1, CTGF, and Colla1* mRNA in mice kidney. (G-K) Protein expression of CTGF, vimentin, α-SMA, p-Smad, and t-Smad was examined by western blot and quantified. Data are presented as means ± S.E.M. N= 4-5 for each group, * p< 0.05, ** p< 0.01, *** p < 0.001, as compared with sham. # p < 0.05, ## p < 0.01, ### p < 0.001 as compared with indicated group.

**Figure 4. AST-120 treatment modulated inflammation and UPRs in AKI**

(A-C) Protein expression of BiP and CHOP in mice kidneys was examined by western blot and quantified. Data are presented as means ± S.E.M. N=4-5 for each
group. (D-E) Correlation efficiency of serum IS-BiP and IS-CHOP level represented as Pearson's $r$. (F-H) Quantification of mRNA levels of senescence-associated secretory phenotype factors: $IL-6$, $TNF-\alpha$, $PAI-1$. Data are presented as means ± S.E.M. N=4-5 for each group. * p<0.05, ** p<0.01, *** p < 0.001, as compared with sham. # p < 0.05, ## p < 0.01, as compared with indicated group.

**Figure 5. IS potentiated cell stress after Hypoxia / Reperfusion (H/R) in HK2 cells.**

(A-C) Cell cycle distribution was measured by flow cytometry. Data are presented as means ± S.E.M. N = 6, *** p < 0.001, as compared with control. ##, p < 0.05, ### p < 0.001, as compared with indicated group. (D) Cellular reactive oxygen species were detected by DCFDA / H2DCFDA. Scale bar indicates 200μm. (E) Protein expression of Histone H2Ax phosphorylation was examined by western blot and quantified. (F-I) IS treatment potentiated EMT-related proteins after H/R. Protein expression of CTGF, E-cadherin, and α-SMA in HK-2 treated with or without IS H/R was examined by western blot and quantified. (J-M) IS treatment potentiated unfolding protein responses (UPR) after H/R. Protein expression of BiP, eIF2α phosphorylation, and CHOP was examined by western blot and quantified. Data are presented means ± S.E.M. N=5 for each group, ** p < 0.01, *** p < 0.001, compared with control, # p < 0.05, compared with indicated group.
Figure 6. IS potentiated cell cycle arrested, and EMT progression can be attenuated by chemical chaperone 4-PBA treatment.

(A-B) Protein expression of BiP and eIF2α phosphorylation in HK-2 treated with or without 4-PBA, IS and H/R was examined by western blot and quantified. (C-D) Protein expression of E-cadherin and α-SMA was examined by western blot and quantified. Data are presented means ± S.E.M. N= 4-6 for each group, * p < 0.05, ** p < 0.01, *** p < 0.001 compared with control. # p < 0.05, ## p < 0.01, ### p < 0.001 as compared with indicated group. (E-F) G2/M phase arrest can be rescue by 4-PBA treatment. Data are presented means ± S.E.M. N= 5-6 for each group, *** p < 0.001 compared with control, ### p < 0.001 compared with IS + H/R + 4-PBA group.

Figure 7. The pathological roles of protein-bound uremic toxins IS in post-ischemic AKI-related renal fibrosis.

Schematic diagram depicting the mechanisms of IS accumulation potentiated post-AKI to CKD transition. An increased serum IS level are caused by loss of OAT1 expression in renal epithelial tubular cells in the mouse model of UIRI. AST-120 administration actually reduced serum IS levels and tubulointerstitial fibrosis (TIF) caused by UIRI combined with high protein intake mimics (indole). For the underlying mechanisms, IS exposure potentiated H/R-induced G2/M cell cycle arrest, ER stress, ROS production, and EMT. In conclusion, early uremic toxins elimination
is likely to be a useful strategy in the prevention and/or treatment of post-AKI fibrosis.
