Indoxyl sulfate inhibits proliferation of human proximal tubular cells via endoplasmic reticulum stress

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Kawakami T, Inagi R, Wada T, Tanaka T, Fujita T, Nangaku M. Indoxyl sulfate inhibits proliferation of human proximal tubular cells via endoplasmic reticulum stress. Am J Physiol Renal Physiol 299: F568–F576, 2010. First published June 9, 2010; doi:10.1152/ajprenal.00659.2009.—Uremic toxins can deteriorate renal function, but little is known about their mechanism. Because tubular injury is central to progression of chronic kidney disease (CKD), we investigated the effects of a representative uremic toxin indoxyl sulfate (IS) on tubular cells. IS induced endoplasmic reticulum (ER) stress in cultured human proximal tubular cells, demonstrated by the increase in C/EBP homologous protein (CHOP) in the immunoblots. Moreover, administration of an oral adsorbent AST-120 reduced serum IS concentration and decreased tubular expression of CHOP in immunohistochemistry in 5/6-nephrectomized, CKD model, rats. Furthermore, we disclosed that IS inhibited proliferation of tubular cells in 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and 5-bromo-2′-deoxyuridine assay, whereas the results of trypan blue exclusion and lactate dehydrogenase assay showed that IS did not promote cell death. This inhibition was mitigated by small interfering (si) RNA against CHOP. Furthermore, IS increased the cyclin-dependent kinase inhibitor p21WAF1/CIP1 (p21). Surprisingly, this was mediated by the inflammatory cytokine interleukin (IL)-6, the expression of which was decreased by siRNA against activating transcription factor 4, another ER stress marker; however, the induction of IL-6 and p21 by IS was not suppressed by siRNA targeted to CHOP, suggesting that they were downstream of ER stress, but independent of CHOP. Moreover, we found that their upregulation was dependent on ERK, using the ERK pathway inhibitor U-0126. Collectively, we demonstrated that IS induced ER stress in tubular cells and inhibited cell proliferation via two pathways downstream of ER stress, namely CHOP and ERK-IL-6-p21. These are possible targets for suppressing progression of CKD.

Uremic toxins are compounds that accumulate in patients with renal dysfunction and exert adverse effects on diverse cells and tissues (33). Renal insufficiency causes uremia through retention of uremic toxins, especially in patients with end-stage renal disease. However, it is now thought that even a small deterioration of renal function causes accumulation of uremic toxins, and that they deteriorate cells and tissues in earlier stages of chronic kidney disease (CKD). Therefore, uremic toxins can cause renal injury and promote progression of CKD.

Indoxyl sulfate (IS) is a representative uremic toxin that is known to deteriorate renal function in animal models with CKD (9). Furthermore, the recent Carbonaceous Adsorbent’s Effectiveness Against Progression of Chronic Kidney Disease (CAP-KD) trial showed that the oral adsorbent AST-120 suppressed decline of creatinine clearance of patients with CKD (2). An amino acid tryptophan from diet protein is metabolized into indole by intestinal flora, including Escherichia coli. Indole is absorbed from the intestine and metabolized into IS by the liver. IS is secreted into bile, reaches the intestine, and finally enters the enterohepatic circulation. The main pharmacodynamic mechanism of AST-120 is thought to be adsorption of IS in the intestinal tract, which hinders reabsorption of IS into the circulation. Therefore, the results of the CAP-KD trial suggest an import role of IS in progression of CKD.

Tubular injury is both a marker and a mediator of CKD progression (25, 34). Hence, it is hypothesized that IS might cause injury in tubular cells and advance CKD. Indeed, Nometo et al. (9) reported that IS decreased viability of murine proximal tubular cells. Motojima et al. (24) reported that IS upregulated plasminogen activator inhibitor-1 (PAI-1) through oxidative stress in human proximal tubular cells. These results suggest causative roles of uremic toxins in further aggravating renal injury. However, its biological effects on tubular cells and their underlying mechanisms are not fully known.

Endoplasmic reticulum (ER) stress has been a subject of great interest in pathophysiology of kidney diseases (15). ER stress can result from a number of its disturbances, including oxidative stress, hypoxia, and glucose deprivation (36). ER stress in renal tubular cells has been shown to play a critical role in acute kidney injury in humans and in an animal model of ischemia-reperfusion injury (3, 18). We previously demonstrated that renal tubular cells suffer from ER stress under pathological conditions with proteinuria, which is not only a marker of CKD but also a mediator of CKD progression (27). ER stress is also induced by several nephrotoxic agents, including acetaminophen (21), cisplatin, gentamicin (32), cyclosporine A (31), and heavy metals (14). Our recent study demonstrated that ER stress induces autophagy in tubular cells (16).

ER stress causes the accumulation of misfolded proteins in the ER, which in turn invokes a response conserved throughout eukaryotes called the unfolded protein response (UPR) (42). The UPR emanates intricate downstream signaling pathways and results in the expression of a variety of genes. Some of them, including ER chaperones like glucose-regulated protein (GRP) 78 and GRP94, contribute to maintenance of cellular homeostasis against the stress, but others, including C/EBP homologous protein (CHOP), a transcription factor also known as growth arrest and DNA damage gene-153, are involved in cell cycle arrest and cell death (4, 30). UPR is also involved in inflammatory responses (46).
In the present study, we investigated whether IS induces ER stress in renal tubular cells. We also investigated its effects on cell proliferation and death, and their underlying mechanisms.

**MATERIALS AND METHODS**

Reagents and antibodies. IS, N-acetylcyesteine, tunicamycin, and recombinant human interleukin (IL)-6 were purchased from Sigma (St. Louis, MO). Indoleacetic acid (IA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). U-0126 was purchased from Calbiochem (San Diego, CA). Immunoblot analysis was done using rabbit polyclonal primary antibodies against CHOP, p21 (Santa Cruz Biotechnology, Santa Cruz, CA), pan-actin (Sigma), activating transcription factor (ATF) 4 (Proteintech Group, Chicago, IL), phosphorylated extracellular signal-regulated kinase (ERK) 1/2 and total ERK1/2 (Cell Signaling Technology, Beverly, MA), and goat polyclonal primary antibodies against human IL-6 (R&D Systems, Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated goat antibody against rabbit IgG (Bio-rad Laboratories, Hercules, CA) and HRP-conjugated donkey antibodies against goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

**Cell culture and treatments.** HK-2 cells (CRL-2190) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM/F-12 (1:1) medium (Invitrogen, Carlsbad, CA) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. We dissolved IS in serum-free medium, filtered it for sterilization for each experiment, and stimulated cells with the medium containing IS, while we changed complete medium to serum-free medium for control cells. As for other reagents, control cells were treated with the respective vehicle(s).

**Immunoblot analysis.** After removal of the medium, treated cells were washed three times with ice-cold PBS, scraped off with lysis buffer containing 2% Nonidet P-40, 0.2% SDS, 50 mM Tris buffer (pH 7.4), 150 mM sodium chloride and a protease inhibitor cocktail, Complete Mini (Roche Diagnostics, Mannheim, Germany), incubated on ice for 20 min, then centrifuged at 18,000 g for 10 min at 4°C. The supernatant was used as a whole cell protein lysate. Cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with Tris-buffered saline (pH 7.4) with 0.5% (vol/vol) Tween 20 containing 5% skim milk, probed with the primary antibody against the target protein and a secondary HRP-conjugated antibody in sequence, and developed with chemiluminescence reagents (ECL Plus, GE Healthcare, Buckinghamshire, UK). Equal loading and transfer of proteins among lanes were verified by immunoblot analysis detecting actin. Band intensities were quantified by densitometric analysis using the National Institutes of Health (NIH) Image J software (version 1.38x).

**5/6-Nephrectomized rats.** We performed all animal experiments in accordance with the NIH guidelines for use and care of laboratory animals, and the study was approved by the local ethical committees. Male Sprague-Dawley rats at the age of 8 wk were purchased from Japan Charles River (Yokohama, Japan). After right nephrectomy (1 wk), we ligated the posterior and one or two anterior branches of the left main renal artery and made infarction of approximately two-thirds of the left kidney. After the nephrectomy (12 wk), they were randomly divided into two groups (either vehicle or AST-120 administration). After 4 wk of treatment, they were killed under ether anesthesia. The kidneys were fixed with neutral-buffered formalin. Serum IS levels were measured by a mobile phase, 5% tetrahydrofu ran/0.1 M KH2PO4 (pH 6.5), at a flow rate of 1 ml/min and fluorescence detection (excitation 295 nm and emission 390 nm) using HPLC (Shimadzu, Kyoto, Japan) as described previously (29).

**Immunohistochemistry.** After antigen retrieval by autoclaving in the citrate buffer, neutral-buffered formalin-fixed, paraffin-embedded kidney sections (3 μm) were probed with anti-CHOP (Santa Cruz Biotechnology), biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), and HRP-conjugated avidin (Vector Laboratories).

Development was performed with 3,3’-diaminobenzidine tetrahydrochloride (Wako, Osaka, Japan), followed by counterstaining with methyl green. CHOP staining was scored by estimation of the percentage of tubules that included CHOP-positive nuclei in randomly selected cortical fields under ×200 magnification in a blinded manner.

**3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay.** To quantify cells, we performed 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter 96 AQueous One Solution (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, 4 × 10^4 HK-2 cells were grown in 100 μl of medium on 96-well plates for 48 h. After they were treated as indicated, 20 μl of MTS reagent were added to each well of the 96-well plate. After incubation at 37°C for 1 h, the absorbance at 492 nm was measured using a plate reader (Asys Hitech, Eugendorf, Austria).

**Lactate dehydrogenase assay.** After removal of the medium, treated cells in a 24-well plate were lysed with 0.5% Triton X-100 and incubated at room temperature for 30 min. Lactate dehydrogenase (LDH) concentration of medium and cell lysates was measured using LDH Kit (Kainos Laboratories, Tokyo, Japan) according to the manufacturer’s protocol. The ratio of medium LDH to the sum of medium LDH and cellular LDH was used as a marker of cellular injury.

**Trypan blue exclusion assay.** Treated cells in a six-well plate were incubated with 200 μl of Trypsin-EDTA, added with 200 μl of PBS and pipetted enough. After the cell suspension was mixed with an equal amount of 0.4% trypan blue solution and incubated at room temperature for 1 min, stained (dead) and nonstained (viable) cells were counted using a hemocytometer.

**5-Bromo-2-deoxyuridine assay.** To quantify cell proliferation, 5-bromo-2-deoxyuridine (Brdu) assay (ELISA) was performed using the Brdu Labeling and Detection Kit III (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. Briefly, we added Brdu to the culture medium of treated cells in a 96-well plate and incubated cells for 3 h. Thereafter, cells were fixed and treated with nuclease to partially digest cellular DNA. Cells were then incubated with peroxidase-conjugated antibody to Brdu. Finally, peroxidase substrate was added to yield colored product, and the absorbance at 405 nm (and at 492 nm for a reference) was measured using a plate reader (Asys Hitech).

**Small-interfering RNA.** CHOP-specific and ATF4-specific small-interfering (si) RNAs were purchased from Sigma. The following sequences were used: CHOP1 sense sequence, 5’-CCAGGAAACCGGA-AACAGTT-3’; antisense sequence, 5’-CUCGUGUUCCGUUUCC-UUGTT-3’; CHOP2 sense, 5’-GGUGCUACUGUACCCUCC-3’; antisense, 5’-GGUGCUACUGUACCCUCC-3’; AT4-1 sense sequence, 5’-AGAUCUCCUUUAGUUAUAT-3’; antisense sequence, 5’-UUAACUAAAGGAUAGUCUUT-3’; AT4-2 sense sequence, 5’-GGCUACAGUGCUUGAUAUACUAU-3’; antisense sequence, 5’-UUAACAGAGCUUGUAUCGCU-3’; siPerfect Negative Control was also obtained from Sigma. HK-2 cells were plated in 96-well plates for MTS assay, while they were plated in 60-mm dishes for harvesting proteins. After cells were plated (1 day), transfection with siRNA was carried out using Lipofectamine 2000 and Opti-MEM I Reduced-Serum Medium (Invitrogen). Medium including siRNA was changed to complete medium 6 h after transfection. After transfection (24 h), cells were stimulated as indicated.

**Quantitative real-time PCR.** Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Total RNA (0.5 μg) was reverse-transcribed into cDNA using Superscript II (Invitrogen). Quantitative real-time (qRT) PCR was performed with SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) on an iCycler system (Bio-Rad) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. PCR was performed using the following conditions: 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The primers used were as follows: p1 forward 5’-GGCCAAACGTTGAGATCC-3’ and reverse 3’-GCGUCCACAAAGUAAUCA-5’.
5'-TTCTCCTGCGCATTC-3', p16 forward 5'-GGGTCCGGTGAAGGACATTCG-3', p18 forward 5'-AGTCCGGTGAAGGACATTCG-3' and reverse 5'-TCATTTTCAAGGAGGCCGAGCTC-3', p19 forward 5'-AGTTCCTGGTGAAGCACACG-3' and reverse 5'-TCATTTTCAAGGAGGCCGAGCTC-3', p21 forward 5'-TGTCCCTCCCCCTTGCTTTC-3' and reverse 5'-AAATCCGTCGACTCCGACC-3', GAPDH forward 5'-TCAGCCGCATCTTCTTTTG-3' and reverse 5'-AAATCCGTCGACTCCGACC-3'.

Statistical analysis. All values are expressed as means ± SD. Data for two groups were analyzed using a two-tailed Student’s t-test, and those for more than two groups were compared using ANOVA. Differences with P values < 0.05 were considered significant.

RESULTS

Induction of ER stress by IS in tubular cells. To investigate whether IS induces ER stress in renal tubular cells, we incubated HK-2, human proximal tubular cell line, cells in serum-free medium including IS and performed immunoblot analysis of the representative ER stress marker CHOP. As shown in Fig. 1A, CHOP was increased by IS up to 8 h and then declined. Furthermore, IS dose-dependently increased CHOP (Fig. 1B). These results demonstrate that IS induces ER stress in proximal tubular cells.

We also examined whether IS induces ER stress in tubular cells in vivo, using CKD model animals, 5/6-nephrectomy rats that were treated with or without the oral adsorbent AST-120 to reduce the serum IS concentration. In fact, the serum IS concentration of AST-120-treated animals was significantly lower than that of control (0.087 ± 0.015 vs. 0.450 ± 0.086 mg/dl, P < 0.01, n = 6). We evaluated ER stress in the kidneys using immunohistochemistry of CHOP. As shown in Fig. 1C and D, CHOP-positive nuclei were conspicuous in cortical tubules of the control kidneys compared with those of AST-120-treated animals. The ratio of tubules with CHOP-positive nuclei was significantly higher in control rats than that in AST-120-treated ones (56.7 ± 4.2 vs. 42.4 ± 5.2%, P < 0.01) (Fig. 1E). These results suggest that IS causes ER stress in tubules in vivo.

However, the concentration of IS that induced ER stress in tubular cells in vitro (2 mM) was much higher than the plasma concentration of IS in patients with end-stage renal failure (250 μM) (26). We hypothesized that the whole effects of many uremic toxins could cause ER stress in tubular cells in vivo. In fact, another uremic toxin, IA, which per se induced ER stress in HK-2 cells, had additive effects with IS (Fig. 1F), supporting our hypothesis.

Involvement of reactive oxygen species in the ER stress induction. It has been reported that IS produces reactive oxygen species (ROS) in this proximal tubular cell line (24) and other cells (12, 43) and that ER stress is closely related to oxidative stress (22). Therefore, we hypothesized that oxidative stress might mediate ER stress by IS. As shown in Fig. 2, the antioxidant N-acetylcysteine remarkably suppressed the increase in CHOP by IS, indicating that IS induced ER stress via ROS production, namely oxidative stress.

Fig. 1. Induction of endoplasmic reticulum (ER) stress by indoxyl sulfate (IS) in tubular cells. A: HK-2 cells were treated with IS for the indicated time. Immunoblots of C/EBP homologous protein (CHOP) are shown. Actin was used as an equal loading control. B: tubular cells were treated with the indicated dose of IS for 8 h. Immunoblots of CHOP are shown. The induction of ER stress by IS was confirmed by the increase in CHOP. C and D: immunohistochemistry of CHOP in the renal cortex of chronic kidney disease (CKD) model rats treated with vehicle (C) or AST-120 (D). E: the ratio of tubules with CHOP-positive nuclei in immunohistochemistry is shown. **P < 0.01 compared with the control. F: HK-2 cells were treated with the indicated dose of IS and/or indoleacetic acid (IA) for 8 h. Immunoblots of CHOP are shown.
Inhibition of tubular cell proliferation by IS. To assess the effects of IS on viability of tubular cells, we performed MTS assay, in which the absorbance is in proportion to the viable cell number. Twenty-four hour treatment with IS resulted in a dose-dependent decrease in tubular cell number (Fig. 3A). Notably, significant decline was seen in 2 mM or over of IS, where ER stress was markedly induced (Fig. 1B). This illustrates that the effect was mediated by ER stress.

It is possible that IS reduced cell number 1) by promoting cell death or 2) by inhibiting cell proliferation. To determine whether IS leads to cell death or not, we carried out LDH assay in which LDH leakage from cells into medium is used as a marker of cellular damage. As shown in Fig. 3B, IS did not enhance LDH release. In addition, the results of trypan blue exclusion assay, where only dead cells are stained by the pigment, also show that tubular cell death was not enhanced by IS (Fig. 3C). The number of viable cells that we counted in the same assay coincides with the results of MTS assay (Fig. 3, A and D).

Because it is unlikely that IS promotes tubular cell death, we performed BrdU assay to evaluate cell proliferation. Indeed, IS significantly suppressed tubular cell proliferation (Fig. 3E). Taken together, IS did not accelerate cell death, but inhibited cell proliferation.

Implication of CHOP in growth suppression by IS. CHOP is a transcription factor that is induced by ER stress (30) and plays a role in cell growth arrest (4). Accordingly, we speculated that CHOP induced by IS might lead to suppression of cell proliferation. To test this hypothesis, we used siRNA targeted to CHOP. Two siRNAs against CHOP significantly reduced induction of the protein by IS (Fig. 4A).

Furthermore, both siRNAs significantly mitigated suppression of cell growth by IS in MTS assay (the siRNAs, CHOP1 and CHOP2, reduced the decrease of absorbance by 44 and 64%, respectively) (Fig. 4B), which directly demonstrates that ER stress per se led to inhibition of cell proliferation.

Induction of p21 by IS through upregulation of IL-6. Cell cycle is intrinsically regulated by cell cycle regulatory proteins, including cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors (38). Because CDK inhibitors play an important role in blocking cell cycle progression, we investigated whether they are involved in tubular cell growth suppression provoked by IS. p21, a CDK inhibitor, was upregulated by IS at mRNA (Fig. 5A) and protein (Fig. 5B) levels. This was not due to serum deprivation, because p21 was not induced in control cells (Fig. 5C). Other CDK inhibitors, including p15, p16, p18, and p19, were not increased in qRT-PCR (data not shown). These results suggest that p21 induction by IS contributes to hindrance of cell proliferation.

It has been reported that p21 is induced by the inflammatory cytokine IL-6 in some cell lines (5, 10) and that ER stress is associated with inflammatory responses (46). Hence, we investigated whether IL-6 is involved in p21 upregulation. Indeed, immunoblot analysis showed that IS increased expression of IL-6, which preceded upregulation of p21 (Fig. 5, A and B). Furthermore, treatment with IL-6 significantly enhanced p21 in tubular cells (Fig. 5E). These results indicate that IS induces the cell cycle regulator p21 through IL-6 expression.
Involvement of ER stress in the IL-6-p21 pathway. To investigate whether ER stress is related to the IL-6-p21 pathway elicited by IS, we used the representative ER stress-inducer tunicamycin. Indeed, treatment with this agent enhanced both IL-6 and p21 in tubular cells, where the increase in IL-6 was followed by that in p21 as observed in IS-treated cells (Fig. 6A), suggesting the relevance of ER stress.

ATF4 is induced by ER stress and constitutes the UPR (35). Because it is reported that ER stress induces IL-6 through ATF4 in endothelial cells (11), we tested whether ATF4 is engaged in the upregulation of IL-6 by IS to further show the involvement of ER stress. First, we confirmed that IS remarkably increased ATF4 (Fig. 6B), which is followed by the expression of IL-6 (Fig. 5B). In addition to CHOP, the expression of ATF4 verifies that IS induces ER stress in tubular cells. Two siRNAs against ATF4, the effectiveness of which are shown in Fig. 6C, significantly attenuated IL-6 induction by IS (Fig. 6D), which clarifies the involvement of ER stress. In contrast, knockdown of CHOP with siRNA did not suppress induction of IL-6 or p21 by IS (Fig. 6, E and F). These results suggest that the IL-6-p21 pathway is induced by ER stress, but independent of CHOP.

An essential role of ERK in the IL-6-p21 pathway. We previously reported that ER stress activates ERK, a mitogen-activated protein kinase, in proximal tubular cells (16). In addition, because ERK is reported to promote IL-6 production in proximal tubular cells (19), we examined whether ERK was involved in IL-6 upregulation by IS. To ascertain this hypothesis, we used the ERK pathway inhibitor U-0126. U-0126 abrogated the increase in IL-6 in immunoblots (Fig. 7A). Furthermore, this ERK pathway inhibitor also blocked p21 induction by IS (Fig. 7B). Upregulation of CHOP was not inhibited by U-0126 (data not shown), indicating that CHOP was independent of ERK and that the effect of U-0126 was not due to attenuation of ER stress induced by IS. We assessed ERK activation, using immunoblots of phosphorylated, namely activated, ERK. Indeed, U-0126 completely blocked the increase of phosphorylated ERK (Fig. 7C). Taken together, the IL-6-p21 pathway elicited by IS was dependent on ERK phosphorylation.

DISCUSSION

We demonstrated that the uremic toxin IS induces ER stress through oxidative stress in human proximal tubular cells and inhibits cell proliferation. Furthermore, ER stress suppresses cell proliferation via, at least, two downstream pathways, namely CHOP and the ERK-IL-6-p21 pathway.

The primary finding of this study is that the uremic toxin IS elicits ER stress in tubular cells. Uremic toxins have a variety of adverse effects on many kinds of cells and tissues. Among a variety of uremic toxins, homocysteine is known to cause ER stress in endothelial cells (28), and hyperhomocysteinemia is linked to atherosclerosis, especially in patients with renal dysfunction (8, 13). Furthermore, not only IS but other uremic toxins can elicit oxidative stress (37), which is closely related to ER stress. Hence, ER stress may be a common mechanism through which uremic toxins lead to cellular injury.

The number of known uremic toxins has been expanding as a new toxin is found one after another. There are so many uremic toxins, including assumed undiscovered ones, that the whole effects of toxins could cause ER stress in tubular cells in vivo. This may explain why the concentration of IS that induced ER stress (2 mM) in cultured tubular cells was higher than that in plasma of patients with end-stage renal failure (250 μM) (26). Indeed, we showed the additive effect of IS and another uremic toxin IA on the induction of CHOP in tubular cells.

We examined biological effects of IS on tubular cells and found that IS does not induce tubular cell death but leads to inhibition of cell proliferation. Because CKD advances through tubular cell injury, its regeneration plays an important role in mitigating CKD progression. Thus tubular cell growth suppression resulting from IS retention in CKD can accelerate further progression of CKD, making a vicious cycle.

Furthermore, we found that IS increased IL-6 in tubular cells. Other groups also reported that IS elevated transforming
growth factor-β1 (23) and PAI-1 (24). These factors produced by IS can deteriorate renal function through recruiting inflammatory cells, injuring tubular cells, and leading to renal fibrosis.

ER stress can bring about either cell growth arrest (6, 7) or cell death (42). Similarly, CHOP, a transcription factor induced by ER stress, can result in either consequence, although their mechanisms and the target genes remain to be elucidated (4, 30). In this report, we demonstrated that CHOP elicited by IS directly contributes to inhibition of cell proliferation, using siRNA against CHOP. Thereby, we revealed that ER stress is an essential mediator through which IS leads to growth suppression of tubular cells.

The rise in CHOP expression by IS was transient, but it indeed had an effect on growth suppression of tubular cells, which was shown using siRNA against CHOP. It is not due to insufficient dose because 5 and 20 mM of IS similarly increased CHOP expression (data not shown). Moreover, acetaminophen also induces ER stress and transiently increased CHOP in murine proximal tubular cells (21), consistent with our results.

A few studies mentioned CHOP in the context of CKD. A recent paper reported that CHOP was upregulated in kidneys of aged diabetic mice and that CHOP might aggravate diabetic nephropathy (40). Besides tubular cells, CHOP might be expressed in glomerular cells, inflammatory cells, and interstitial cells. A role of CHOP in progression of CKD remains to be elucidated.

Previous reports suggest that ER stress can initiate inflammatory responses, including IL-6 production (20), and that the level of p21 can be increased or unchanged in ER stress-induced cell growth arrest (6, 7, 45). The representative ER stress inducer tunicamycin, as well as IS, induced IL-6, followed by upregulation of p21 in tubular cells. Furthermore, we showed that ATF4, a transcription factor that is induced by ER stress and brings about UPR, is involved. These results suggest that ER stress per se is involved in their upregulation induced by the uremic toxin.

It is well known that p21 is a potent CDK inhibitor, functions as a regulator of cell cycle progression, and leads to cell cycle arrest (38). In this study, we showed that IS induces ER stress, increases the expression of p21, and inhibits cell proliferation. Similarly, the heavy metal cadmium elevates p21 expression and causes cell cycle arrest in rat proximal tubular cells (41). Furthermore, ER stress is induced by cadmium in tubular cells (44). These suggest that p21 may be a common regulator through which ER stress inhibits renal tubular cell cycle progression.

Furthermore, we found that IL-6 enhanced p21 in tubular cells. Recent studies report that an inflammatory cytokine-CDK inhibitor axis can result in permanent cell growth arrest, namely cellular senescence, in some conditions (1, 17). Because ER stress might result from various uremic toxins, as mentioned above, and ER stress can provoke both inflammatory responses and cell growth arrest, uremic toxins might

Fig. 5. Interleukin (IL)-6-p21 pathway elicited by IS. A: expression of p21 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was examined by quantitative real-time (qRT) PCR. IS significantly increased the expression of p21 at 12 and 24 h (n = 6). B: immunoblots show the increase in p21 and IL-6 in tubular cells treated with IS. C: immunoblots of p21 in cells treated with or without IS for 24 h. D: immunoblots of IL-6 in cells with or without IS for 8 h. E: immunoblots of p21 in tubular cells treated with the indicated dose of IL-6 for 4 h show that 50 ng of IL-6 significantly increased p21. Representative immunoblots are shown. The graph shows the ratios of band intensities of interested proteins to those of actin, standardized to the mean of the control (n = 3). **P < 0.01 and ***P < 0.001 compared with the control.
result in senescence of cells or tissues through an inflammatory cytokine-CDK inhibitor pathway induced by ER stress.

The IL-6-p21 pathway activated by IS was dependent on ERK phosphorylation. ER stress can activate ERK (16, 20), and ERK can cause cell cycle arrest via upregulation of p21 (39). Furthermore, IL-6 production induced by tumor necrosis factor-α depends on ERK in human proximal tubular cells (19). In macrophages, ER stress upregulates IL-6, depending

Fig. 6. Implication of ER stress in the IL-6-p21 pathway. A: the ER stress-inducer tunicamycin upregulated p21 and IL-6 in immunoblots, suggesting that IS induces them through ER stress. B: IS induced another ER stress marker, activating transcription factor 4 (ATF4), with its peak at 4 h, in the immunoblot. C: HK-2 cells treated with each siRNA were exposed to IS for 4 h. Immunoblots of ATF4 validated the effectiveness of both siRNAs against ATF4. D: both siRNAs against ATF4 significantly suppressed the increase of IL-6 by IS. A representative immunoblot is shown. The graph shows the ratios of band intensities of IL-6 to those of actin, standardized to the mean of the control (n = 3). ***P < 0.001 compared with the control (IS 0 mM). #P < 0.05 compared with the negative control. E and F: siRNAs against CHOP did not decrease IL-6 (E) or p21 (F) induced by IS.

Fig. 7. Upstream of the IL-6-p21 pathway: involvement of extracellular signal-regulated kinase (ERK). HK-2 cells were treated with or without 10 μM of U-0126 (an inhibitor of the ERK pathway) in 0 or 5 mM of IS for 8 h (A) or 24 h (B). Immunoblots of IL-6 (A) and p21 (B) are shown. Upregulation of both IL-6 and p21 was significantly suppressed by U-0126, indicating the involvement of ERK. Activation of ERK and its inhibition by U-0126 are shown in immunoblots of phosphorylated (p) ERK, with total (t) ERK as the control (C). Representative immunoblots are shown. The graphs show ratios of band intensities of interested proteins to those of actin (A and B) or total ERK (C), standardized to the mean of the control (n = 3). **P < 0.01 compared with the control without IS. ###P < 0.001 compared with the control without U-0126.
on ERK (20). These reports support our findings of the relevance of ERK.

In conclusion, retention of uremic toxins in CKD can lead to further tubular lesion via tubular cell growth inhibition. ER stress causes this effect through CHOP, inflammatory cytokines, and CDK inhibitors, suggesting that these are possible targets for suppressing CKD progression in the future.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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