博士論文

D-serine, a novel uremic toxin, induces senescence in human renal tubular cells via derangement of serine metabolism

（**D-**セリンは、アミノ酸代謝経路を介して尿細管老化を亢進させる新規尿毒素である）

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Table of contents

1. Abstract · · · · · · 3

2. Introduction · · · · · · 5

3. Materials and Methods · · · · · · 9

4. Results · · · · · · 15

5. Discussion · · · · · · 36

6. Acknowledgements · · · · · · 41

7. Disclosure · · · · · · 42

8. References · · · · · · 43

Abstract

Chronic kidney disease (CKD), characterized by progressive renal dysfunction, is increasing in prevalence due to societal aging. Uremic toxins, accumulated during renal dysfunction, can cause kidney damage, leading to further renal deterioration. A recent metabolome analysis of CKD patients revealed that the accumulation of plasma D-serine was associated with faster progression of renal dysfunction. However, this association has not yet been confirmed and the mechanisms behind its action are still unclear. In the present study, we demonstrated that D-serine markedly suppressed the proliferation of a human proximal tubular cell line, HK-2, and that of primary cells, normal human renal epithelial cells. This effect was caused by the induction of apoptosis, as well as of cellular senescence, which was accompanied by G2/M cell cycle arrest and senescence-associated secretory phenotype, which included the induction of pro-fibrotic and pro-inflammatory factors. These upregulated factors can contribute to tubulointerstitial fibrosis. Intriguingly, we found that integrated stress response mediated by the general control nonderepressible 2 played a central role in the D-serine-induced cell toxicity and the pro-fibrotic phenotypes, all of which are known to accelerate CKD progression and kidney ageing. Importantly, D-serine upregulated the L-serine synthesis pathway, which is a source of L-serine. Furthermore, D-serine-induced suppression of tubular cell proliferation was ameliorated by L-serine

administration, indicating that exposure to D-serine caused an L-serine-deprived state in tubular cells and that L-serine synthesis occurred to compensate for this deprivation. Thus, this study, for the first time, unveiled the molecular mechanisms of D-serine-induced tubular damage and pro-fibrotic phenotypes, suggesting that D-serine might be a uremic toxin involved in the pathogenesis of CKD.

Introduction

Chronic kidney disease (CKD) is a common disease worldwide[1]. It increases the risk of end-stage kidney failure, cardiovascular disease, and even premature death[2], apart from burdening the patients and the society with the cost[3]. However, no definitive treatment is available to overcome this problem so far. Researchers all over the world have been investigating the pathophysiology of CKD, and over the past two decades, CKD research has been performed in the field of "systems biology," ranging from genomics to metabolomics[4]. In the field of chiral amino acid metabolomics, recent technical development has made it possible to distinguish between D- and L-amino acids and revealed the existence of D-amino acids in the living world, suggesting the possibility of studying cell biology based on amino acid chirality [5]. However, the biological functions of D-amino acids or their relevance in CKD are not fully elucidated.

A previous report had revealed that plasma D-serine levels were elevated in aged individuals and patients with CKD[6], while another had reported that the risk of advanced progression to end-stage kidney disease was approximately 3-fold higher in patients with CKD who had the highest levels of plasma D-serine than those who had the lowest levels[7]. These reports were confirmed by our preliminary data, and emphasized the importance of further investigation to understand the CKD pathophysiology based on amino acid chirality. Thus, we studied the link

between D-amino acids and CKD to address the pathophysiological role of D-amino acids in kidney injury. Although D-serine nephrotoxicity has been reported in different species, but it has not been reported in humans. D-serine was used in clinical trials for the treatment of schizophrenia without any reports of renal complications[8-12]. One study reported proteinuria without renal dysfunction in a patient who received high doses of D-serine[13], suggesting the possibility of dose-dependent toxicity of D-serine to the human kidney.

Uremic toxins are a group of compounds that accumulate in proportion to renal dysfunction and exert deleterious effects on cells. They negatively affect cells and tissues in CKD, accelerate renal injury, and promote the progression of CKD[14], thus creating a vicious cycle. Given the fact that D-serine accumulates in proportion to renal dysfunction, it is possible that it is a novel uremic toxin if it harms cells or tissues. It is therefore important to evaluate the biological function of D-serine to understand the pathophysiology of CKD from a novel viewpoint such as amino acid chirality.

Amino acid chirality is very important for some biological functions, especially in stress signals; depletion of some L-amino acids, but not D-amino acids, induces stress signals by activating the general control nonderepressible 2 (GCN2). GCN2 is one of the four eukaryotic translation initiation factor 2 alpha (eIF2a) kinases, the other three being the double-stranded

RNA-dependent protein kinase (PKR), the heme-regulated eIF2a kinase (HRI), and the PKR-like ER kinase (PERK). They converge on the eIF2a phosphorylation to activate the integrated stress response (ISR), which induces the expression of activating transcription factor 4 (ATF4), resulting in apoptosis-related signals such as C/EBP homologous protein (CHOP)[15]. The PERK-dependent ISR, via ATF4 and CHOP, induces the production of pro-inflammatory cytokines and the up-regulation of p21 human renal tubular cells, causing CKD progression[14]. The GCN2-dependent ISR, which is activated in response to amino acid starvation, also contributes to disease progression; the GCN2-dependent ISR has been reported to aggravate pressure overload–induced congestive heart failure[16]. Therefore, in the present study, we hypothesized that the GCN2-dependent ISR has a pathophysiological effect on human renal tubular cells.

CKD has been associated with cellular senescence [17]. In particular, tubular cell cycle arrest is closely linked to tubular senescence, leading to CKD progression[18]. Of note, recent evidence has highlighted the fact that cellular senescence can induce senescence-associated secretory phenotype (SASP), which includes cell growth arrest and secretion of pro-inflammatory cytokines and pro-fibrotic factors[19]. Thus, SASP could also be induced by senescence-associated tubular damage in CKD and be implicated in CKD progression. Given that D-serine is a putative predictive marker of poor prognosis of patients with CKD, we hypothesized that D-serine induces

tubular damage via SASP-associated acceleration of cellular senescence. In the present study, we examined D-serine-mediated toxicity in human proximal tubular cells and its molecular mechanisms; in particular D-serine-induced stress signals were investigated.

Materials and Methods

Cell lines and cell culture

The human proximal tubular cell line HK-2 was cultured in DMEM/F12 supplemented with 5% fetal calf serum (FCS). L-serine and D-serine (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in very small amounts of distilled water and added directly to the medium before use. Normal human renal epithelial cells (NHREC) were purchased from Kurabo (Osaka, Japan) and cultured in Renalife medium (Lifeline Cell Technology, Frederick, MD). The amino acids D-/L-alanine, D-/L-serine and D-/L-proline were purchased from Wako. MK-801 (Wako) and ifenprodil (Sigma-Aldrich) were used as antagonists of NMDARs as reported previously[20].

Reagents and antibodies

Monoclonal mouse anti-p21 (1:200), polyclonal rabbit anti-PERK (phospho T981) (1:200) and anti-PERK (1:200) and polyclonal rabbit anti-ATF6 antibodies were obtained from Santa Cruz; monoclonal mouse anti-CHOP antibody (1:1000) was obtained from Cell Signaling Technology (Danvers, MA, USA). Monoclonal rabbit anti-histone H3 (phospho S10) (1:1000), monoclonal rabbit anti-GCN2 (1:1000) and monoclonal rabbit anti-GCN2 (phospho T899) (1:500) antibodies were obtained from Abcam (Cambridge, UK). Monoclonal mouse anti-phospho-Histone H2A.X

(Ser139) (1:50) was purchased from Millipore (MA, USA). Finally, polyclonal rabbit anti-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO).

Analyses of cell viability, cell cycle progression, and apoptosis

Cells were seeded into 6-well plates at 100,000 cells per well. After 24 h, cultures were incubated with L-serine, D-serine, or both at the indicated concentrations for 48 h in the presence of 5% FCS. Cell number and viability were evaluated using the Muse™ Count & Viability Assay Kit, cell cycle progression by the Cell Cycle Assay Kit and apoptosis by Annexin V staining, the Dead Cell Assay kit and caspase 3/7 activity assays (components of the Muse™ Cell Analyzer; Millipore Corp.) according to the manufacturer's instructions.

Cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium, inner salt (MTS)

Cell viability was estimated by MTS assay using CellTiter 96 Aqueous One Solution reagent (Promega Corp, Madison, WI) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at 3,500 cells per well and incubated with L-serine, D-serine or both in the presence of 5% FCS and the presence or absence of various drugs as indicated. Absorbance at

490 nm was measured on a microplate reader (EnSpire, Perkin-Elmer) 1−3 h after adding 20 μL of the reagent to each well.

Real-time quantitative and semi-quantitative PCR

Cell RNA was isolated using RNAiso Plus (Takara) and reverse transcribed using PrimeScript RT Master Mix (Takara). Synthesized cDNA was used as a template for PCR quantification at 1:40 (vol/vol) in the PCR reaction mixture. PCR was performed on a CFX96 cycler (Bio-Rad, Hercules, CA) with KAPA SYBR Fast Universal 2 qPCR Master Mix (Kapa Biosystems, Wilmington, MA). Relative expression levels were calculated using β-actin mRNA expression as the reference. Primer sequences are listed in Table 1.

Western blot analysis

Cultured cells were lysed by adding lysis buffer (WAKO), and total protein concentration was measured using a Pierce BCA Protein Assay Kit (ThermoFisher). Sample buffer containing 0.25 M Tris-HCl (pH 6.8), 8% sodium dodecyl sulfate (SDS), 20% sucrose, 5% β-mercaptoethanol and 0.02% bromophenol blue was added to the lysate and proteins were separated on 7.5%−15% SDS polyacrylamide gels. Separated proteins were then transferred onto polyvinylidene difluoride

transfer membranes (GE Healthcare, Buckinghamshire, UK) in a Tris-glycine transfer buffer (48 mM Tris-buffer, 39 mM glycine, 0.05% SDS and 10% methanol). Membranes were incubated with primary and secondary antibodies as indicated, and an ECL Plus Western Blotting System (GE Healthcare) was used for detection. Reproducibility was confirmed in at least three independent experiments, and representative blots are presented in the figures. Band intensity was quantified using ImageJ (National Institutes of Health).

Senescence-assosicated β -galactosidase staining

100,000 cells of NHREC were plated in 6-well dishes, cultured for 4 days in media with or without 20 mM D-serine, and stained with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to detect SA-βG activity in the fashion described previously[21]. Briefly, the cells were washed with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS. After removal of the fixation solution, the cells were incubated with SA-βG staining solution for 16 h, washed with PBS and methanol, and examined under microscope (model BZ-9000; Keyence). At least 7 randomly taken areas were evaluated to determine the percentage of SA-βG positive cells.

Immunocytochemistry

20,000–40,000 cells (HK-2 or NHREC) were cultured in chamber slides (SCS-008; Matsunami, Osaka, Japan) and fixed for 5 min with solution consisting of ice cold 70% methanol and 30% acetone. Then cells were incubated with the primary antibodies for 16 hours and subsequently incubated with fluorescence-conjugated secondary antibody for 1 hour in darkness. Hoechst 33258 (1:10,000; Sigma-Aldrich) was used for the detection of nucleus. Fluorescent images were captured using a BZ-9000. At least 7 randomly taken areas were evaluated to determine the percentage of cells positive for immunofluorescence.

Cytokine release

Human IL-6 and IL-8 levels were measured by chemiluminescent enzyme immunoassay (R&D Systems) and homogeneous time resolved fluorescence (Cisbio Bioassays), respectively, according to the manufacturers' instructions.

RNA interference

Gene expression was knocked down using targeted siRNAs against human GCN2 (sc-45645, Santa Cruz), ATF4 (sc-35112, Santa Cruz) and CHOP (ThermoFisher, Cat. 1299001). Results were compared against a supplier-matched control siRNA (sc-37007 from Santa Cruz and Cat. 12935112

from ThermoFisher). These siRNAs were introduced into HK-2 cells during passage using Screenfect A reagent (Wako) according to the manufacturer's instructions.

Statistical Analysis

All data are reported as mean ± SEM. Two groups were compared by independent sample t-test and multiple groups by ANOVA with the post hoc Bonferroni tests for pair-wise comparisons. A $P < 0.05$ was considered statistically significant for all tests. GraphPad Prism software, version 5.04 for Macintosh (GraphPad Software, San Diego, CA) was used for data analyses.

Results

D-serine, but not L-serine, suppressed proliferation and induced apoptosis in human tubular cells

To assess the pathophysiological effects of D-serine on human tubular cells, we first investigated the effect of D-serine on the proliferation rates of an immortalized human proximal tubular cell line, HK-2, and normal human renal epithelial cells (NHREC). When these cells were treated with various doses (0-20 mM) of D- or L-serine for 24 or 48 h, we found that D-serine, but not L-serine, markedly reduced the cell proliferation rate in a dose-dependent manner, as measured by the MUSE Cell Count & Viability Assay and MTS assay, (Fig. 1A-1D). Furthermore, Annexin V staining and caspase 3/7 activity analysis revealed that D-serine (20 mM for 48 h) induced more apoptosis in HK-2 cells than controls without serine, while L-serine showed no such apoptotic effect (Fig. 1E, 1F). This tubular cell apoptosis after D-serine treatment was associated with increased expressions of pro-apoptotic genes, *BAX* and the p53-upregulated modulator of apoptosis (*PUMA*) in both HK-2 and NHREC, and decreased expressions of anti-apoptotic gene *BCL-2*, in NHREC, although *BCL-2* expression only showed tendency to decrease in HK-2 (Fig. 1G, H). These results demonstrated that D-serine induced mitochondria-dependent apoptosis resulting in a reduction of viable tubular cells. Such tubular toxicity was not observed in other D-amino acids such as D-alanine and D-proline,

both of which are also reported to be accumulated during kidney dysfunction (Fig. 1I).

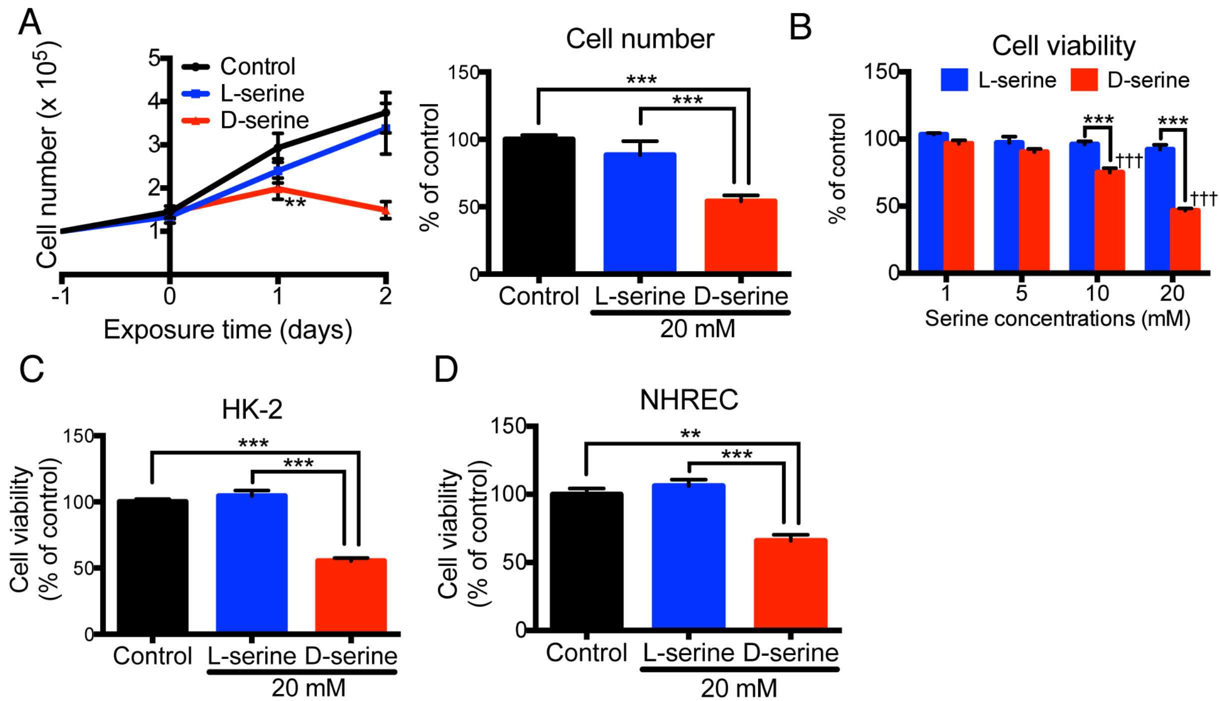

Figure 1. D-serine suppresses cellular proliferation and induces apoptosis in human tubular cells

(A) HK-2 cells were grown in a normal medium (DMEM/F12 containing 5% FBS) and treated with 20 mM L-serine or D-serine for 48 h. Cell number was measured by the Muse Count & Viability Kit, which contains fluorescent dyes that distinguish live from dead cells. Each bar represents the average of control, D-serine-treated, or L-serine-treated cultures (n =6). On the left graph, *P < 0.01, ***P < 0.001. compared to untreated control cells. ††† P < 0.001 compared to D-serine-treated cells.

(B-D) HK-2 cells (B, C) or normal human renal epithelial cells (NHREC) (D) were treated with L- or D-serine at 1-20 mM (B) or 20 mM (C, D). Viability was assessed using MTS (n = 4 cultures for each treatment groups)

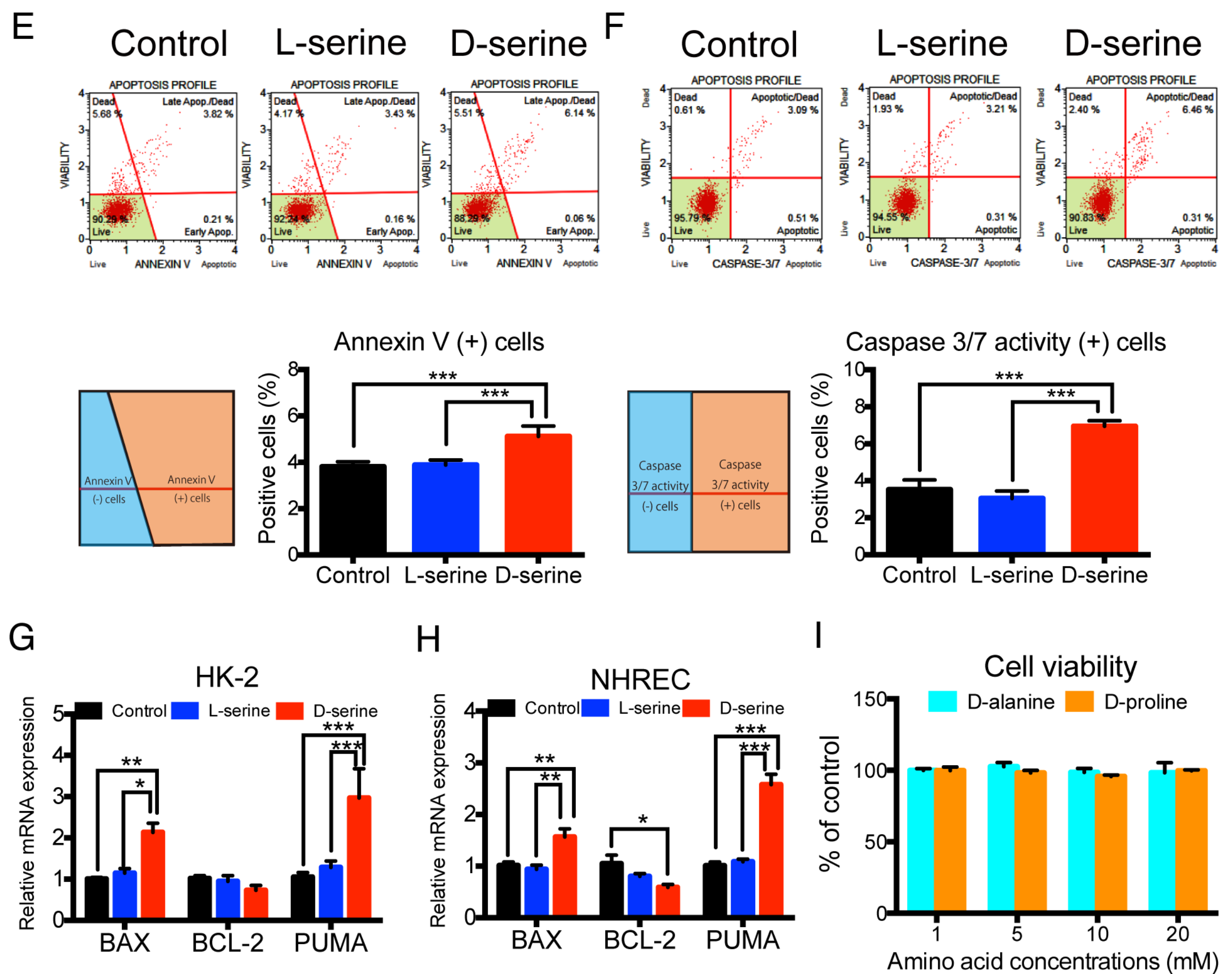

Figure 1(cont.).

(E) Apoptosis induced by D-serine in HK-2 cells as shown by Annexin-V binding and propidium iodide (PI) staining. HK-2 cells treated with 20 mM D-serine for 48 h showed a significant increase in Annexin V staining compared with that of control cells and cells treated with 20 mM L-serine (n = 3 cultures per treatment group).

(F) Activation of caspase 3/7 by D-serine but not L-serine (n = 3 cultures per treatment group).

(G, H) Increased expression of pro-apoptotic BAX, and PUMA in HK-2 cells (G) and NHREC (H) and decreased expression of anti-apoptotic BCL-2 in NHREC (H) treated with D-serine for 48 h (n = 6 cultures per treatment group).

(I) HK-2 cells were treated with D-alanine or D-proline at 1-20 mM for 48 h and viability was assessed using MTS (n = 4 cultures for each treatment groups).

*P < 0.05, **P < 0.01, ***P < 0.001. All data expressed as mean ± SEM.

D-serine induced G2/M cell cycle arrest in human tubular cells

The reduction in the cell proliferation rate by D-serine was greater than the increase in the proportion of apoptotic cells (Fig. 1A, 1E-F), suggesting the D-serine-mediated tubular cell arrest. Indeed, a propidium iodide (PI)-based cell cycle assay revealed that D-serine induced cell cycle arrest at the G2/M stage in HK-2, whereas L-serine did not (Fig. 2A). Phosphorylation of histone H3, which is a known G2/M marker[22], was also significantly increased in D-serine-treated HK-2 (Fig. 2B). To confirm these results, we compared the expression of p21, a cell cycle inhibitor, in tubular cells with or without D-serine by real-time PCR and Western blot, and confirmed that p21 was upregulated by D-serine in these cells at mRNA and protein levels (Fig. 2C-E).

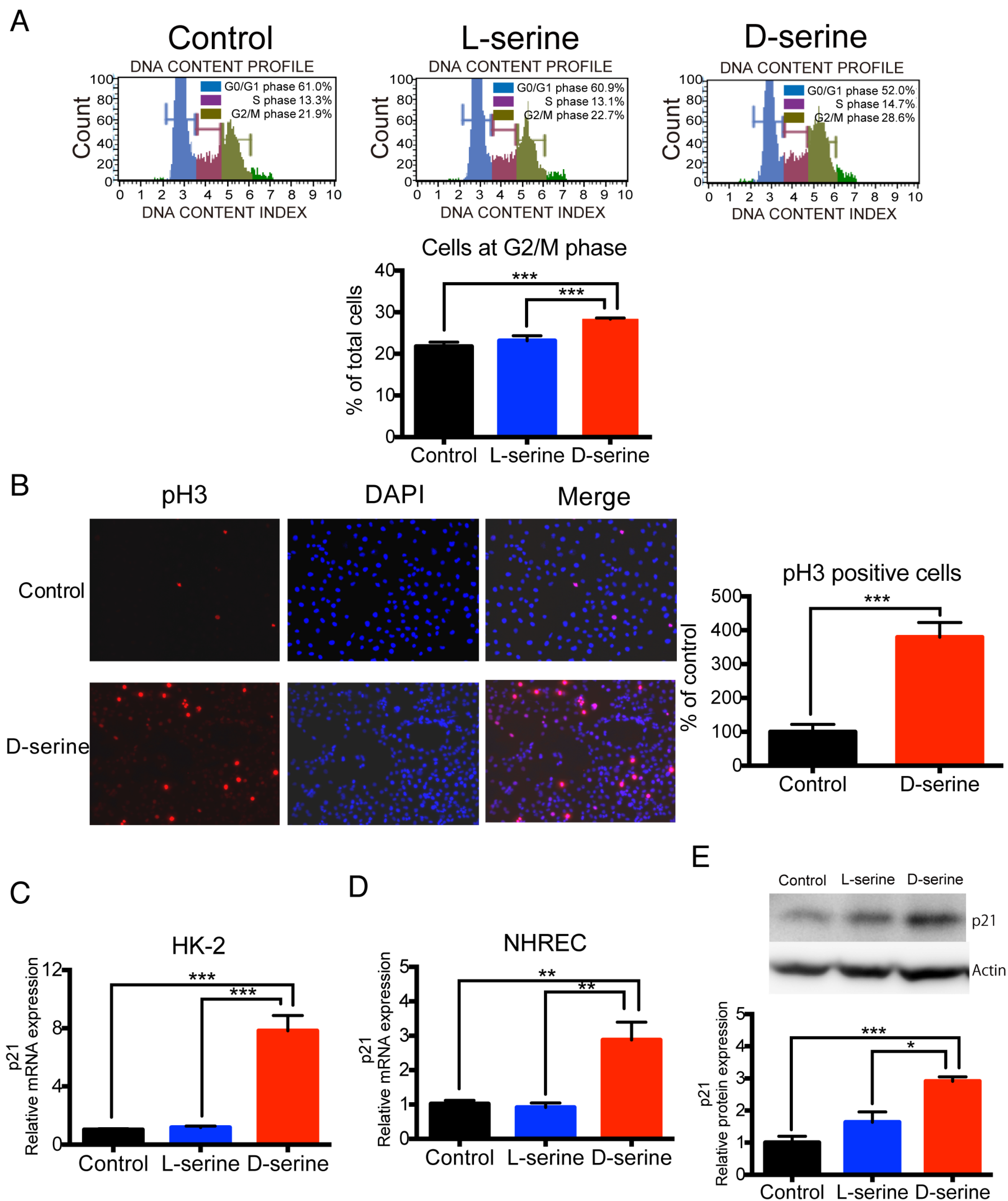

Figure 2. D-serine induces cell cycle arrest at the G2/M phase.

(A) Cell cycle analyses by PI staining after 48 h of treatment with L- or D-serine indicated that D-serine induced cell cycle arrest at G2/M (n = 3 cultures per treatment group).

(B) Representative images immunofluorescence staining of pH3. Quantification of the percentage of cells positive for pH3 with exposure with or without 20 mM D-serine (n = 7 areas taken randomly per treatment group)

(C, D) Upregulation of p21 in HK-2 cells (C) and NHREC (D) treated with 20 mM D-serine for 48 h as determined by qRT-PCR (n = 6 cultures per treatment group).

(E) Western blot for p21 expression. Increased expression of p21 was confirmed at the protein level in HK-2 cells (n = 3 cultures per treatment group). A typical image and densitometric analysis are shown.

$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All data expressed as mean ± SEM.

D-serine induced tubular senescence with SASP

Previous studies has reported that when injured renal tubular cells are arrested at G2/M, they adopt a pro-fibrotic phenotype, which influences surrounding epithelial cells[18, 23]. After confirming that D-serine caused tubular cell cycle arrest at G2/M, we investigated the fate of arrested cells. First, senescence markers involved in cell cycle inhibition, including p16 and p21 (see Fig. 2C-E), were found to be increased in D-serine-treated tubular cells (Fig. 3A, B). Furthermore, as expected, the D-serine-treated tubular cells showed the senescence phenotypes, which was estimated by cellular senescence markers such as senescence-associated beta-galactosidase (SA-βgal) and γ-H2AX (Fig. 3C, D). Importantly, the D-serine-induced tubular cell senescence was associated with SASP in HK-2 and NHREC. The levels of molecules that support D-serine induced SASP, such as pro-inflammatory cytokine IL-6 and chemokine IL-8, were also enhanced after D-serine treatment at the mRNA and protein levels (Fig. 3E – 3H). These data demonstrated that D-serine induced tubular cell senescence with SASP.

Cells adopting the SASP could also become phenotypes that are damaged or pro-fibrotic. To confirm this, we investigated expressions of MMP-9 and VEGF-A, both renal pro-fibrotic markers[24, 25]. The former remodels extracellular matrix (ECM), and the latter induces detachment of pericytes from capillaries and promotes to make them ECM-producing

myofibroblasts. As expected, we observed increased expressions of these markers (Fig. 3I). These results showed that D-serine might be not only pro-inflammatory but also pro-fibrotic.

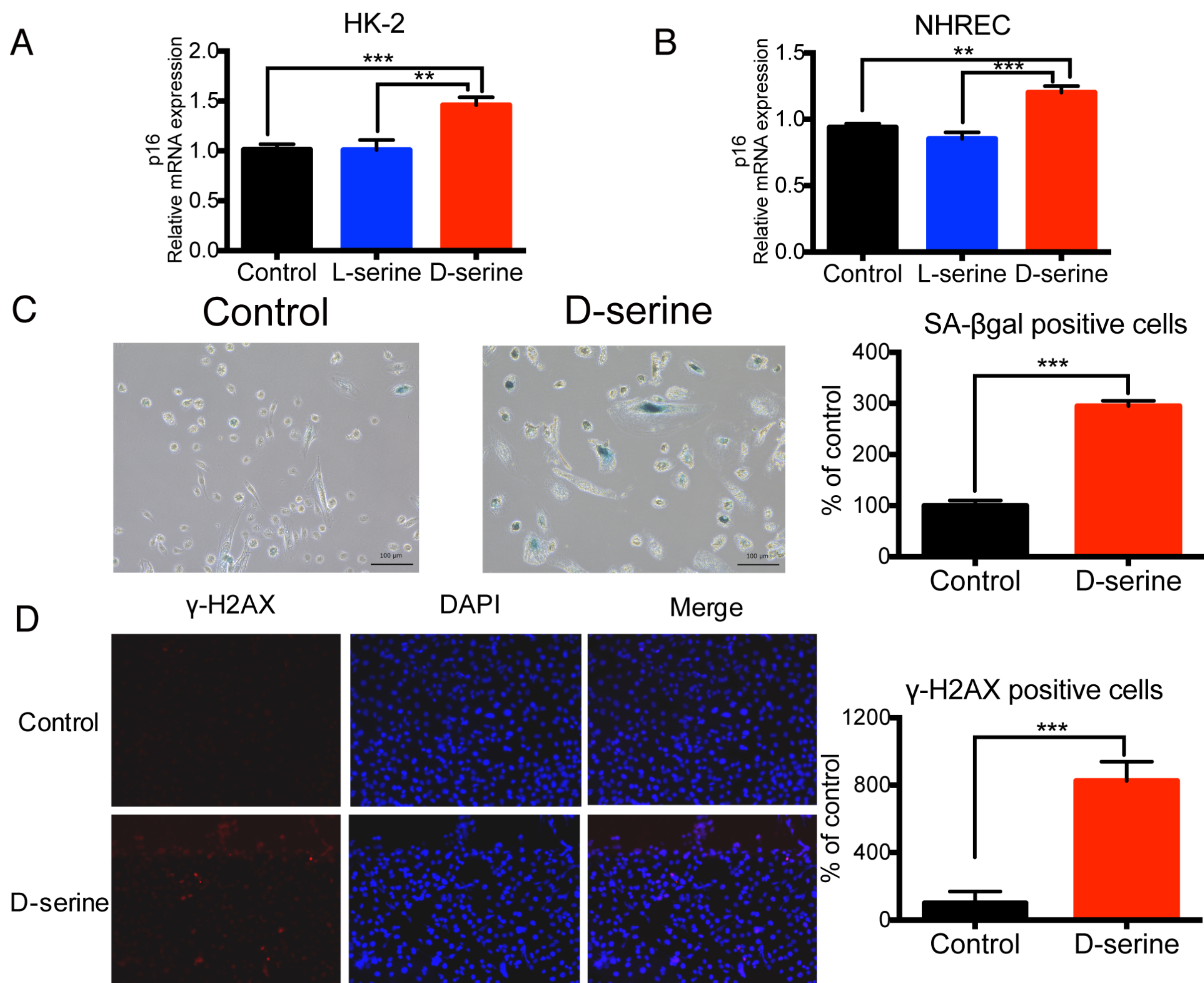

Figure 3. D-serine induces tubular senescence with senescence-associated secretory phenotype (SASP)

(A, B) Upregulation of p16 gene expression in HK-2 (A) and NHREC (B) treated with 20 mM D-serine for 48 h (n = 6 cultures per group).

(C) Representative pictures of SA-βgal staining of control and D-serine treated NHREC and quantitative assessment. (n = 7 areas taken randomly per treatment group)

(D) Representative pictures of immunofluorescence staining of γ-H2AX of control and D-serine treated HK-2 and quantitative assessment. (n = 7 areas taken randomly per treatment group)

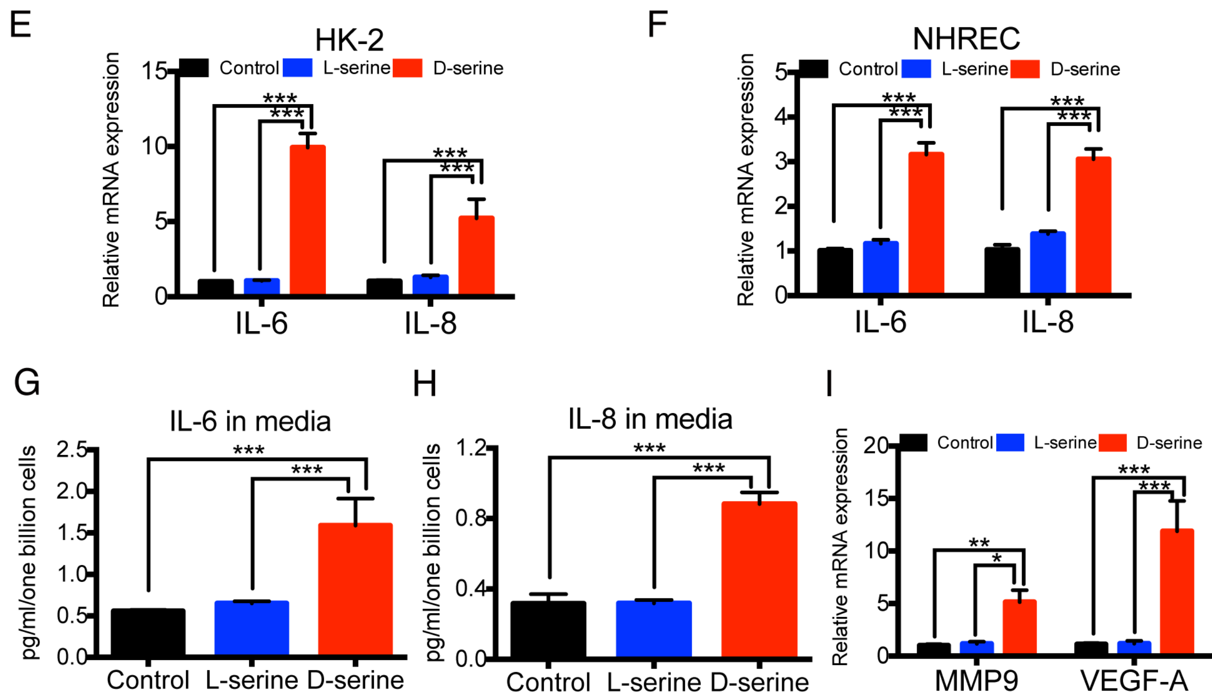

Figure 3 (cont.).
(E, F) Expression levels of a pro-inflammatory cytokine IL-6 and chemokine IL-8, two senescence-associated secretory phenotype (SASP) markers, were upregulated in HK-2 (E) and NHREC (F) by 20 mM D-serine treatment for 48 h (n = 6 cultures per group).
(G, H) D-serine also induced the secretion of IL-6 (G) and IL-8 (H) into the media as measured by ELISA (C, n = 3 cultures per group) and HTRF respectively (D, n = 3 cultures per group) in HK-2.
(I) Expression levels of MMP-9 and VEGF-A, renal pro-fibrotic markers, were upregulated in HK-2 by 20 mM D-serine treatment for 48 h (n = 6 cultures per group).
$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All data expressed as mean ± SEM

D-serine-induced tubular cell senescence was via GCN2 activation as an integrated stress response.

Next, we hypothesized that exposure to D-serine may disrupt amino acid status associated with the activation of ISR, leading to tubular cell senescence. To address this issue, we examined the association between D-serine and GCN2, which is activated by L-amino acid starvation and plays key roles in modulating amino acid metabolic pathway and ISR as a serine/threonine-protein kinase. Indeed, D-serine activated GCN2 in HK-2, as evidenced by the increase in phosphorylation compared to the controls or L-serine-treated cells (Fig. 4A). Importantly, GCN2 activation by D-serine induced the downstream ISR signal pathway; ATF4 and CHOP were upregulated at the mRNA level (Fig. 4B) and CHOP was increased at the protein level as well (Fig. 4C) in D-serine-treated HK-2. A similar pattern of GCN2-mediated ISR activation was also observed in NHREC (Fig. 4D).

Because CHOP is a known effector of ER stress, which is related to ISR, we examined whether ER stress modulators, PERK and ATF6, were also involved in the D-serine-induced ISR. However, PERK and ATF6 were not activated by phosphorylation and cleavage, respectively (Fig. 4E).

To assess the mechanistic contribution of GCN2 activation in tubular cell senescence, we

used siRNA transfection to knock down GCN2. The knockdown of GCN2 expression (Fig. 4F) significantly mitigated the D-serine-induced activation of ISR, as demonstrated by the expression level of ATF4 (Fig. 4G). D-serine-induced SASP was also ameliorated by GCN2 knockdown; D-serine-induced upregulation of IL-6 and IL-8 was markedly decreased, while the expression of p21 was unaffected, or showed a tendency to decrease, in the tubular cells after GCN2 knockdown (Fig. 4G). CHOP, which is activated by GCN2-mediated ISR pathway, was also decreased after CHOP knockdown (Fig. 4H), and D-serine-induced impairment in cell proliferation was significantly ameliorated (Fig. 4I). These data demonstrated that D-serine-induced, GCN2-mediated ISR caused SASP in and impaired cell proliferation of tubular cells.

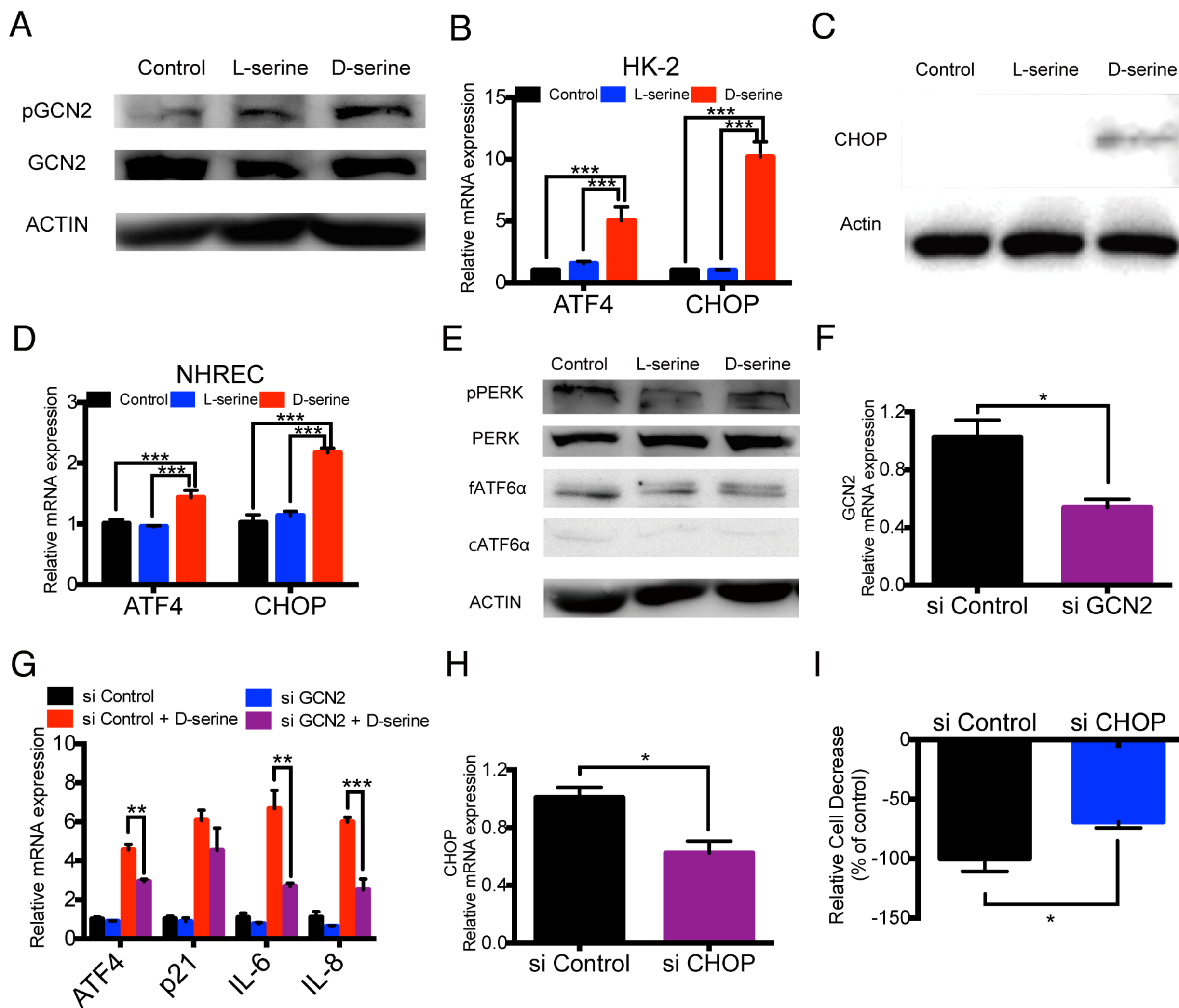

Figure 4. D-serine-induced tubular cell senescence is via GCN2 activation as an integrated stress response.

(A) D-serine (20 mM for 48 h) phosphorylated GCN2 in HK-2.

(B) D-serine also upregulated the ISR signaling molecules ATF4 and CHOP in HK-2 (n = 6 cultures per treatment group).

(C) D-serine exposure increased CHOP protein expression compared with that in control and L-serine-treated HK-2.

(D) D-serine exposure (20 mM for 48 h) upregulated ISR-related molecules in NHREC (n = 6 cultures per group).

(E) Western blotting analysis showed no change in PERK, phosphorylated PERK or cleaved ATF 6α expression in D-serine-treated HK-2.

(F) siRNA-mediated GCN2 knockdown induced down-regulation of the GCN2 gene (n = 4 cultures per treatment group).

(G) siRNA-mediated GCN2 knockdown suppressed the upregulation of ATF4, IL-6 and IL-8 in D-serine-treated HK-2 (n = 4 cultures per treatment group).

(H) siRNA-mediated CHOP knockdown induced down-regulation of the CHOP gene in HK-2 (n = 4 cultures per treatment group).

(I) siRNA-mediated CHOP knockdown ameliorated the cell proliferation impairment in HK-2 using MTS (n = 4 cultures per treatment group).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All data expressed as mean ± SEM.

D-serine activates L-serine synthesis pathway and D-serine-induced tubular toxicity was altered by D/L-serine ratio

Considering the possibility that D-serine deranges the amino acid status, we next examined the changes in the L-serine synthesis pathway. The regulation of L-serine synthesis pathway (Fig. 5A) has recently attracted great interest in the field of cancer research, because L-serine starvation is known to upregulate the senescence marker, p21, as well as the GCN2-dependent ISR[26], thus promoting its downstream signals, leading to apoptosis and cell cycle arrest. To examine if D-serine induces L-serine starvation and thus enhances L-serine synthesis, we first assessed the expression levels of enzymes important for the synthesis of L-serine from glucose. The enzymes hexokinase (HK), phosphofructokinase 1 (PFK1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphoglycerate kinase 1 (PGK1), and L-serine synthesis pathway enzymes, phosphoglycerate dehydrogenase (PHGDH), and phosphohydroxythreonine aminotransferase 1 (PSAT1), were all significantly upregulated after a 48-h D-serine treatment (Fig. 5B). Furthermore, D-serine slightly, but significantly, augmented glucose consumption, leading to the production of L-serine (Fig. 5C). Since PHGDH and PSAT1 are known to be regulated by ATF4[27], and to be extremely important for the L-serine synthesis pathway[28, 29], we knocked down ATF4 to confirm that

GCN2-mediated ISR induced the L-serine synthesis pathway. Upon knockdown of ATF4, we observed down-regulation of both PHGDH and PSAT1 genes (Fig. 5D).

After thus confirming the upregulation of the L-serine synthesis pathway, we investigated the ability of L-serine to rescue the renal tubular cells from D-serine-induced toxicity. Surprisingly, the addition of no more than 1 mM L-serine completely reversed the D-serine-induced decrease in HK-2 cell numbers (Fig. 5E). In contrast, the addition of other L-amino acids did not ameliorate the effects of D-serine (Fig. 5E), suggesting that this ability to reverse D-serine-induced toxicity was not generic for L-amino acids, but specific to L-serine. The expression levels of effector molecules, such as p21, CHOP, and IL-8, which were induced by D-serine treatment, were also rescued by treatment with 1 mM L-serine in both HK-2 and NHREC (Fig. 5F, G). When L-serine was removed in the medium, the D-serine-induced toxicity was augmented in HK-2 cells (Fig. 5H; also compare the D-serine dose–responses in Fig. 1A to Fig. 5H). Furthermore, alteration of the D/L-serine ratio modified the deleterious effects of 20 mM D-serine on cell survival (Fig. 5I).

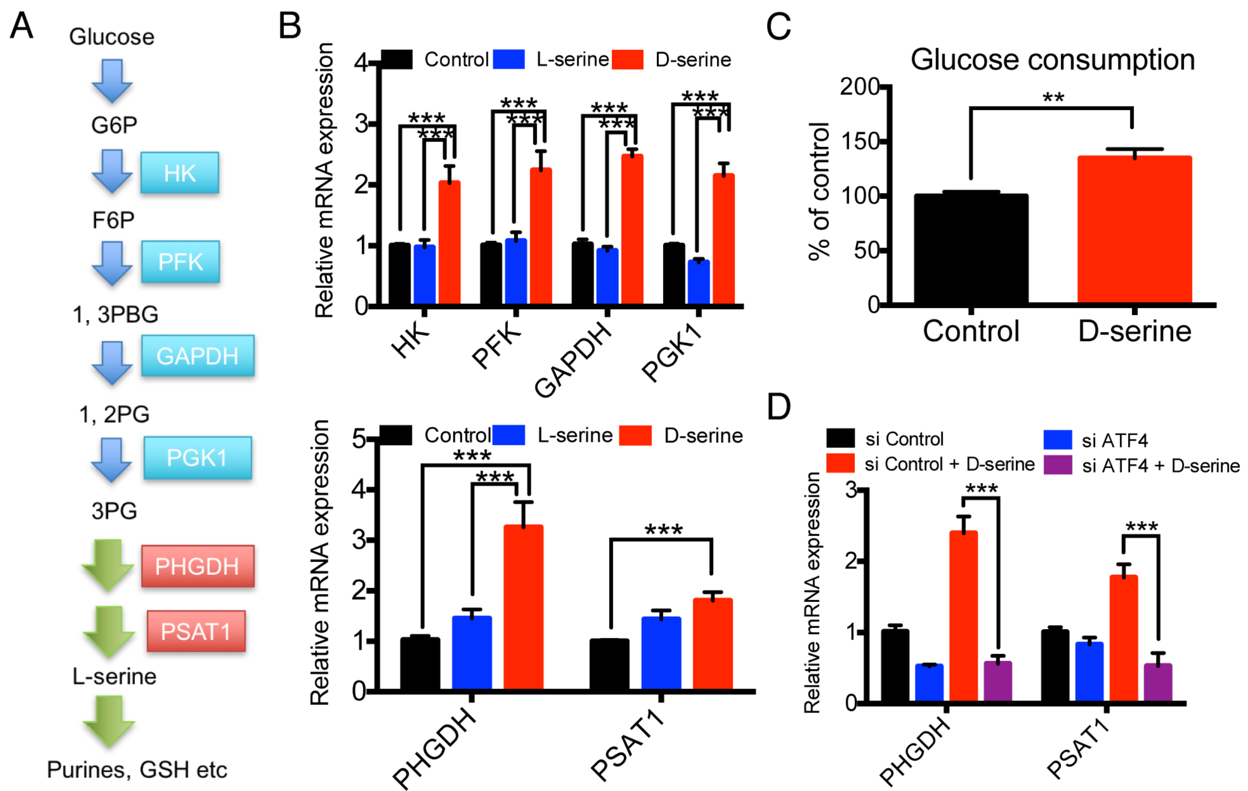

Figure 5. D-serine activates L-serine synthesis pathway and D-serine-induced tubular toxicity is altered by D/L-serine ratio.

(A) The L-serine synthesis pathway from glucose, with important enzymes.

(B) Expressions of enzymes involved in the L-serine synthesis pathway were upregulated at the mRNA level by D-serine (20 mM for 48 h) in HK-2 (n = 6 cultures per treatment group).

(C) Glucose consumption was enhanced by 20 mM D-serine (n = 3 cultures per treatment group).

(D) siRNA-mediated ATF4 knockdown suppressed the upregulation of PHGDH and PSAT1 genes in D-serine-treated HK-2 (n = 4 cultures per treatment group).

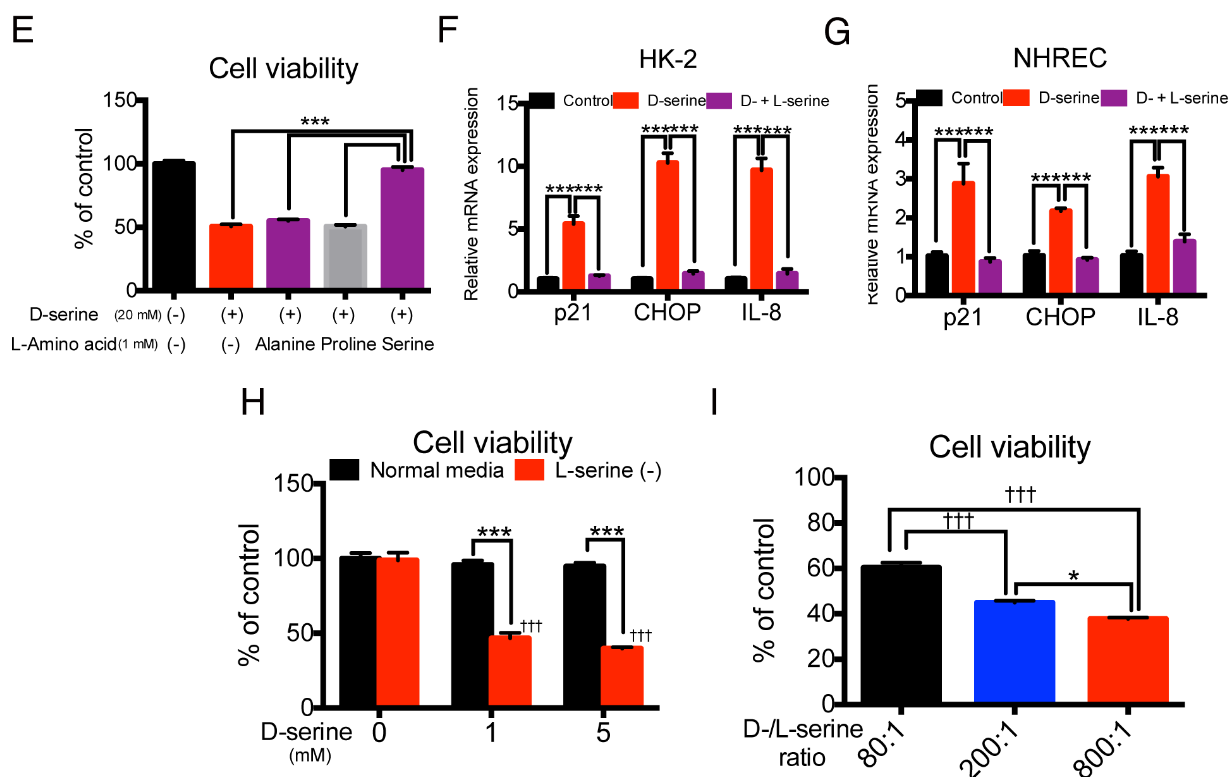

Figure 5 (cont.).

(E) L-serine (1 mM) completely reversed the reduction in HK-2 cell viability induced by 20 mM D-serine, while L-alanine or L-prolibe did not (n = 4 cultures per treatment group).

(F, G) L-serine (1 mM) completely reversed the increased expressions of p21, CHOP, and IL-8 induced by 20 mM D-serine in HK-2 (F) and NHREC (G) (n = 4 cultures per treatment group).

(H) The D-serine-induced cell toxicity (20 mM) was enhanced using MTS assay when using L-serine free media in comparison with when using normal media (L-serine 0.25 mM) in HK-2 (n = 4 cultures per treatment group). †††P < 0.001 compared to control cells.

(I) D-serine cell toxicity increased in proportion with D/L-serine ratio in the presence of 20 mM D-serine (n = 4 cultures per treatment group). †††P < 0.001 compared with control cells.

*P < 0.05, **P < 0.01, ***P < 0.001. All data expressed as mean ± SEM.

D-serine-induced cell decrease was not related to NMDAR activation.

D-serine is a strong co-agonist in NMDA receptor (NMDAR)[30], and NMDAR expression in the kidneys was already confirmed[31]. To confirm NMDAR was not involved in D-serine-induced toxicity, we performed NMDAR inhibition together with D-serine treatment in HK-2 by a non-competitive inhibitor, MK-801, or Glun1R specific antagonist, ifenprodil, did not ameliorate cell number reduction, or an ISR signal, an apoptotic marker, or a SASP marker up-regulation induced by D-serine (Figure 6A, B).

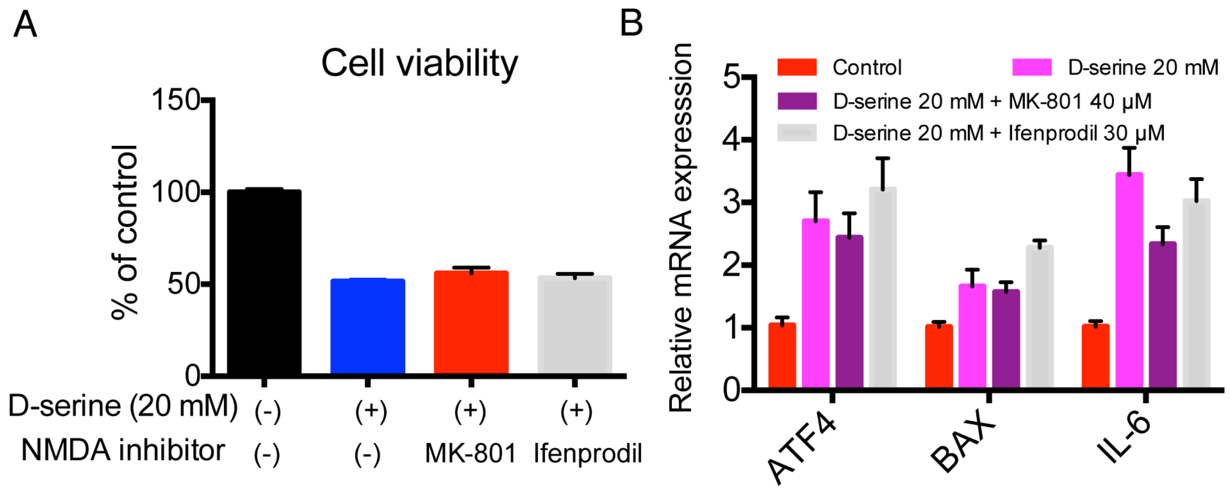

Figure 6. D-serine-induced cell toxicity is not ameliorated by NMDA inhibitors.
(A) Cell viability of HK-2 after 48-h exposure of 20 mM was not ameliorated by 40 µM of MK-801 or 30 µM of ifenprodil using MTS assay (n = 4 cultures per treatment group).
(B) NMDA inhibitors were not effective in rescuing the increased expressions of ATF4, BAX, and IL-6 induced by 20 mM D-serine at the mRNA levels in HK-2 (n = 4 cultures per treatment group).

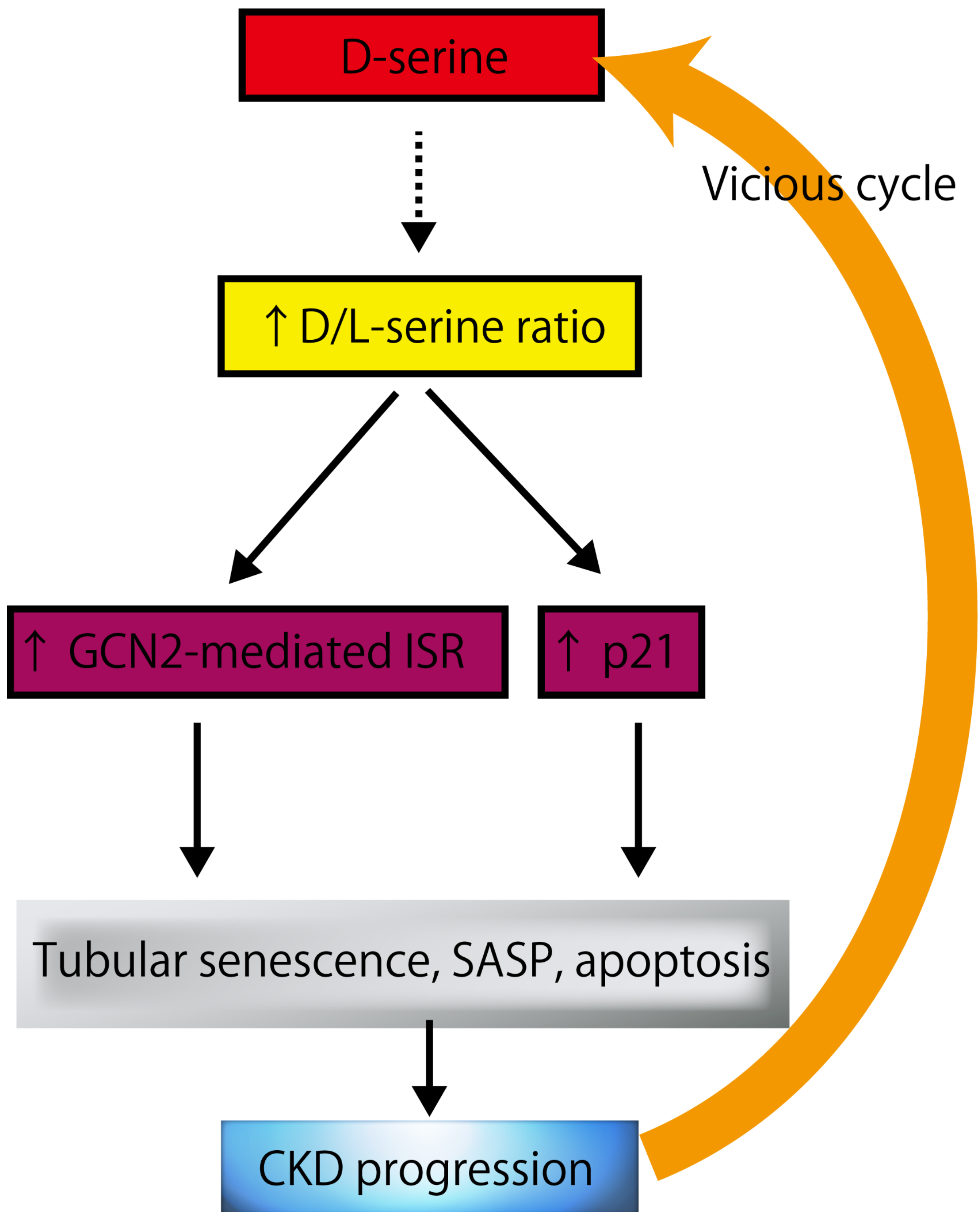

Figure 7. D-serine accelerates CKD via activation of ISR and p21.
High concentrations of D-serine and D/L-serine ratios alter stress related to amino acid homeostasis. This in turn activates p21 and GCN2, which upregulates ATF4, resulting in cell cycle arrest and apoptosis. Tubular cell senescence leads to CKD progression. With increased CKD severity, plasma D-serine concentration rises, creating a vicious cycle of progressively greater D-serine toxicity and renal failure.

Table 1. Nucleotide sequences of the primers used for qRT-PCR in this study

Genes	Primer Sequence
Human ß-actin	Forward 5′-TCCCCCAACTTGAGATGTATGAAG-3′ Reverse 5′-AACTGGTCTCAAGTCAGTGTACAGG-3′
Human bax	Forward 5′-TGGAGCTGCAGAGGATGATTG-3′ Reverse 5′-CCCAGTTGAAGTTGCCGTCAG-3′
Human bcl-2	Forward 5′-TGGGAGAACAGGGTACGATA-3′ Reverse 5′-CATCTCCCGCATCCCACTC-3′
Human PUMA	Forward 5′-GGTCCTCAGCCCTCGCTCTC-3′ Reverse 5′-CTTGTCTCCGCCGCTCGTAC-3′
Human p21	Forward 5′-GTGGCCTTGTCGCTGTCTT-3′ Reverse 5′-GCGCTTGGAGTGATAGAAATCTG-3′
Human p16	Forward 5′-GGGGGCACCAGAGGCAGT-3′ Reverse 5′-GGTTGTGGCGGGGGCAGTT-3′
Human IL-6	Forward 5′-GGTACATCCTCGACGGCATCT-3′ Reverse 5′-GTGCCTCTTTGCTGCTTTCAC-3′
Human IL-8	Forward 5′-AAGGAAAACTGGGTGCAGAG-3′ Reverse 5′-ATTGCATCTGGCAACCCTAC-3′
Human MMP9	Forward 5′-CACTGTCCACCCCTCAGAGC-3′ Reverse 5′-GCCACTTGTCGGCGATAAGG-3′
Human VEGF-A	Forward 5′-ATCTGCATGGTGATGTTGGA-3′ Reverse 5′-GGGCAGAATCATCACGAAGT-3′
Human ATF4	Forward 5′-GTTCTCCAGCGACAAGGCTA-3′ Reverse 5′-ATCCTCCTTGCTGTTGTTGG-3′
Human CHOP	Forward 5′-TGCTTTCAGGTGTGGTGATGTA-3′ Reverse 5′-AATCAGAGCTGGAACCTGAGGA-3′
Human GCN2	Forward 5′-GAAGCTGTCAGCCAGCACTA-3′ Reverse 5′-GTTGGCAAGGGAGGTCTGAA-3′
Human HK	Forward 5′-GGCTCATTTCCACCTCACCA-3′ Reverse 5′-GGAGGGCAGCATCTTAACCA-3′
Human PFK	Forward 5′-TGAAGCCAGAGAGGCCTTAGA-3′ Reverse 5′- GGAACCAGGGAGAGATGTGC-3′
Human GAPDH	Forward 5′-CCTCAACGACCACTTTGTCA-3′ Reverse 5′-TTACTCCTTGGAGGCCATGT-3′
Human PGK1	Forward 5′-CTGTGGCTTCTGGCATACCT-3′ Reverse 5′-GCTGCTTTCAGGACCACAGT-3′
Human PHGDH	Forward 5′-ATCTCTCACGGGGGTTGTG-3′ Reverse 5′-AGGCTCGCATCAGTGTCC-3′
Human PSAT1	Forward 5′-CGGTCCTGGAATACAAGGTG-3′ Reverse 5′-AACCAAGCCCATGACGTAGA-3′

Discussion

In this study we demonstrated that a novel uremic toxin, D-serine, had highly deleterious effects on human renal proximal tubular cells in vitro. Exposure to D-serine led to cellular senescence with G2/M cell cycle arrest, and SASP, accompanied with enhanced apoptosis. Furthermore, the D-serine-induced tubular cell toxicity upregulated pro-fibrotic factors, and might thus cause tubulointerstitial fibrosis. This was initiated by the activation of GCN2, a sensor of amino acid status and ISR initiator [26, 32, 33]. Low concentrations of serum D-serine are normally maintained by kidney through excretion[34]; we suggest that D-serine toxicity initiates kidney dysfunction and thus increases D-serine levels, in a vicious cycle of elevated D-serine levels, leading to progressively greater kidney senescence and nephrotoxicity (Fig. 7), ultimately resulting in CKD.

The activation of GCN2, usually provoked by L-amino acid starvation, causes the upregulation of ATF4 and CHOP, leading to cell cycle arrest[35]. This set of these responses is called the ISR. The ISR converges ATF4 activation with activation of GCN2, PKR, HRI, or PERK[15]. GCN2 initially senses an increase in uncharged tRNA, which is considered a signal of L-amino acid starvation. Most studies on GCN2 activation by L-serine starvation have focused on cancer cells or fibroblasts [36], and not cells in mature and healthy organs. In cancer cells, the depletion of L-serine causes impaired cell proliferation via GCN2-dependent ISR[26]. However,

when we cultured HK-2 cells without L-serine, no changes in viability were observed (Fig. 5H). This difference between HK-2 and cancer cells may be attributed to the much higher level of metabolism in the latter. Nonetheless, we demonstrated that D-serine could activate GCN2 in healthy kidney tubular cells as well as cancer cells.

PERK-dependent ISR can be ameliorated by the knockdown of effector molecules, especially CHOP, in human tubular cells[14]. In this study, GCN2-dependent ISR was ameliorated by the siRNA-mediated knockdown of CHOP, an important ISR effector molecule in ISR. This knockdown reversed the D-serine-induced reduction in number of viable cells. The knockdown of GCN2 also reduced the expression of ATF4 and SASP markers, IL-6 and IL-8 (a pro-inflammatory cytokine and a chemokine, respectively), indicating that blocking the ISR could mitigate the toxic effects of D-serine on renal tubular cells. Therefore, it is possible that the progression of CKD could be slowed down by the administration of ISR inhibitors.

Prolonged exposure to D-serine upregulated the molecules involved in ISR, inducing cellular senescence via SASP and apoptosis. D-serine also concomitantly induced the L-serine synthesis pathway (as evidenced by increased glucose consumption and upregulation of multiple enzymes of the L-serine synthesis pathway). Two possible scenarios could explain these phenomena: one involves D-serine-induced cell toxicity, while the other involves the tubular cells

somehow being inaccessible to L-serine. Considering the fact that D-serine-induced tubular cell toxicity depends on D-/L-serine ratio and that the damage induced by D-serine was exaggerated by the deficiency of L-serine (Fig. 5H, I), the latter seems more probable.

The physiological and pathophysiological functions of D-serine have been studied most thoroughly in the central nervous system (CNS), where D-serine is known to act as a co-agonist of N-methyl-D-aspartate receptors (NMDARs) [30]. Aberrant D-serine metabolism has been implicated in a number of neurological and psychiatric diseases such as stroke and schizophrenia [37]. NMDARs are also expressed in the kidneys[31, 38], although their function in the kidneys remains unclear. NMDA antagonists have been shown to protect the kidney cells from oxidative stress[39], and the activation of LPS-mediated toll-like receptor[40]. Thus, the toxic effects of D-serine might have involved the activation of NMDARs. Indeed, blocking the activation of NMDARs in the kidneys is emerging as a promising therapeutic strategy. However, D-serine alone does not activate NMDARs and NMDAR antagonists failed to protect against D-serine-induced toxicity (Fig. 6). Therefore, it is unlikely that the D-serine-induced toxicity reported in the current study is associated with NMDAR activation.

This study has several limitations. First, we have reported only in vitro results using high concentrations of D-serine. Unfortunately, in-vivo studies are difficult to perform as D-serine is

quickly removed from the systemic circulation [41]. The mechanism by which D-serine enters cells and is distributed within the cells also remains unclear. While it is currently difficult to measure intracellular and extracellular D-serine, a feasible method has been recently developed [42]. In future studies, it would be important to directly demonstrate the accumulation of D-serine in organelles. Finally, the concentration of D-serine used in this study was much higher than that found in human plasma (1−20 mM vs. 5 μM in healthy subjects and up to 10 μM in patients with end-stage kidney disease) [34] . However, the D/L-serine ratio further increases to about 10% in patients with the progression of CKD[34, 43]. The relative, rather than the absolute, D-serine concentration may thus be more relevant. Indeed, we demonstrated that lower levels of D-serine showed measurable toxicity in the absence of L-serine (Fig. 5H).

In conclusion, we have provided evidence that D-serine induced senescence in renal tubular cells with pro-inflammatory and pro-fibrotic SASP, accompanied by apoptosis. This nephrotoxicity might contribute to the progression of CKD. Aggravated renal dysfunction due to D-serine-induced senescence or death of tubular cells could further increase systemic D-serine by its reduced excretion, resulting in progressive damage to tubular cells and kidneys. Furthermore, the results of this study indicated that higher D/L-serine ratios lead to tubular cell injury, suggesting that the ratio can be utilized as a marker for progressive renal deterioration in CKD patients with

CKD. Thus, we proposed decreasing D/L-serine ratio, reducing the absolute concentration of D-serine and blocking the downstream effector mechanisms of ISR as potential therapeutic strategies against CKD.

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Competing Interests

No competing interests are declared.

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