NF-κB plays an important role in indoxyl sulfate-induced cellular senescence, fibrotic gene expression, and inhibition of proliferation in proximal tubular cells

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Shimizu H, Bolati D, Adijiang A, Mutelieu G, Enomoto A, Nishijima F, Dateki M, Niwa T. NF-κB plays an important role in indoxyl sulfate-induced cellular senescence, fibrotic gene expression, and inhibition of proliferation in proximal tubular cells. Am J Physiol Cell Physiol 301: C1201-C1212, 2011. First published August 10, 2011; doi:10.1152/ajpcell.00471.2010.—We previously demonstrated that indoxyl sulfate induces senescence and dysfunction of proximal tubular cells by activating p53 expression. However, little is known about the role of nuclear factor (NF)-κB in these processes. The present study examines whether activation (phosphorylation) of NF-κB by indoxyl sulfate promotes senescence and dysfunction in human proximal tubular cells (HK-2 cells). Indoxyl sulfate induced phosphorylation of NF-κB p65 on Ser-276, which was suppressed by N-acetylcysteine, an antioxidant. Furthermore, indoxyl sulfate induced NF-κB p65 expression. Inhibitors of NF-κB (pyrrolidine dithiocarbamate and isohelenin) and NF-κB p65 small interfering RNA (siRNA) suppressed indoxyl sulfate-induced senescence-associated β-galactosidase activity and expression of p53, transforming growth factor (TGF)-β1, and α-smooth muscle actin (SMA). The induction of p53 expression and p53 promoter activity by indoxyl sulfate were inhibited by pifithrin-α, p-nitro, an inhibitor of p53, whereas p53-transfected cells showed enhanced p53 promoter activity. NF-κB inhibitors suppressed indoxyl sulfate-induced p21 expression, whereas NF-κB p65 siRNA enhanced its expression. NF-κB inhibitors partially alleviated indoxyl sulfate-induced inhibition of cellular proliferation. NF-κB p65 siRNA-transfected cells showed less proliferation in the presence of indoxyl sulfate than control cells. Phosphorylated NF-κB p65 was expressed and colocalized with p53, p21, β-galactosidase, TGF-β1, and α-SMA in the kidneys of chronic renal failure (CRF) rats. AST-120, which reduces serum indoxyl sulfate level, suppressed their expression in the CRF rat kidneys. Taken together, NF-κB plays an important role in indoxyl sulfate-induced cellular senescence, fibrotic gene expression, and inhibition of proliferation in proximal tubular cells. More notably, indoxyl sulfate accelerates proximal tubular cell senescence with progression of CRF through reactive oxygen species-NF-κB-p53 pathway.

Chronic renal failure; renal fibrosis; uremic toxin; AST-120

Indoxyl sulfate, a metabolite of tryptophan in dietary proteins, is synthesized in the liver from indole that is generated by intestinal flora including Escherichia coli. Indoxyl sulfate is normally excreted in urine. As renal function deteriorates, however, indoxyl sulfate accumulates in serum due to its reduced renal clearance (26, 27, 32, 33). Accumulated indoxyl sulfate in serum is incorporated into the basolateral membrane of renal proximal tubular cells by mediating organic anion transporter types 1 and 3 (12). Thereafter, indoxyl sulfate accumulates in the cells, thereby inducing nephrotoxicity (12). Administration of indoxyl sulfate and its precursor indole to 5/6 nephrectomized rats increased glomerular sclerosis in the remnant kidneys with a decline in renal function. In addition, indoxyl sulfate induced fibrotic genes such as transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloproteinases-1, and type-I collagen (26, 27). In another experiment, administration of indoxyl sulfate to salt-sensitive hypertensive rats increased the glomerular, mesangial, Masson’s trichrome (MT)-positive tubulointerstitial areas and the expression of glomerular and tubulointerstitial TGF-β1 compared with control rats (1). The accumulation of serum indoxyl sulfate in chronic renal failure (CRF) is also associated with several detrimental effects on thyroxine hepatocyte transport (22), cardiac fibroblasts and myocytes (21), endothelial cells (10, 11, 19, 24, 42, 47), vascular smooth muscle cells (30, 39, 46), aortic calcification (1, 29), and the progression of atherosclerosis (46).

AST-120 is an oral adsorbent that absorbs indole in the intestine and thus decreases the serum level of indoxyl sulfate and consequently blocks indoxyl sulfate accumulation in renal tubules and the progression of renal failure (25). AST-120 suppresses reactive oxygen species (ROS) production (31, 40), cell senescence (2, 38), and fibrosis (25, 38) in the kidney. The senescence markers senescence-associated β-galactosidase (SA-β-gal) and p53 and the fibrosis marker α-smooth muscle actin (SMA) in CRF rats are localized in the renal tubules of CRF rats but are barely detectable in normal rats and in CRF rats treated with AST-120 (38). The indoxyl sulfate-induced activated p53 expression is implicated in the pathway that is connected with cellular senescence and CRF progression, because SA-β-gal activity and α-SMA expression are suppressed by pifithrin-α, p-nitro [PFTα, a p53 inhibitor (38)].

Nuclear factor-κB (NF-κB) is a eukaryotic transcription factor that is normally found in the cytoplasm, where it forms an inactive ternary complex with an inhibitor protein IκB. After appropriate stimuli is applied, IκB is degraded, and NF-κB translocates into the nucleus, where it binds DNA and activates the transcription of target genes. Indoxyl sulfate induces free radical production in proximal tubular cells and thus activates NF-κB that in turn upregulates the expression of plasminogen activator inhibitor-1 (PAI-1) (28). However, little
is known about the other cellular functions of indoxyl sulfate-induced NF-κB activation in proximal tubular cells. Therefore, the present study examines the effects of NF-κB activation by indoxyl sulfate on proximal tubular cell dysfunction and senescence.

MATERIALS AND METHODS

Reagents. Antibodies were obtained from the following suppliers: anti-α-tubulin for immunoblotting, Calbiochem (La Jolla, CA); anti-α-SMA for immunoblotting and immunohistochemistry, Sigma Chemical (St. Louis, MO); anti-p53, anti-p21, anti-phospho-p65 (Ser-276), and anti-TGF-β1 for immunoblotting, as well as anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody and anti-mouse IgG.
HRP-linked antibodies, Cell Signaling Technology (Beverly, MA); anti-phospho-p65 for immunohistochemistry, Abcam (Cambridge, UK); anti-β-gal for immunoblotting, Promega (Madison, WI); and anti-p53, anti-p21, and anti-TGF-β1 for immunohistochemistry, Santa Cruz Biotechnology (Santa Cruz, CA). Indoxyl sulfate was from Alfa Aesar (Lancashire, UK). pNF-κB RE-TK hRluc (F), pGL4-piTP53, pCMV_S-FLAG, and pCMFlag hsTP53 were obtained from the DNA Bank, RIKEN BioResource Center (Ibaraki, Japan). Pyrrolidine dithiocarbamate (PDTC) and isohelenin, inhibitors of NF-κB, PFTα (a p53 inhibitor), and N-acetylcysteine (NAC) (an antioxidant) were from Calbiochem (La Jolla, CA). Dulbecco’s modified Eagle’s medium (DMEM/F12) was purchased from Wako (Osaka, Japan). Trypsin-EDTA, fetal bovine serum (FBS), and insulin-transferrin-selenium were purchased from GIBCO (Grand Island, NY). Penicillin and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan).

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\text{Cell culture. } \text{HK-2 cells purchased from ATCC (Manassas, VA) were maintained in DMEM/F12 supplemented with 10% FBS, insulin-transferrin-selenium, 100 U/ml penicillin, and 100 \mu\text{g/ml streptomycin.}}
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\text{Preparation of siRNAs specific to NF-κB p65 and p21. Small interfering RNAs (siRNAs) specific to NF-κB p65 and p21 and a negative control were obtained from Nippon EGT (Tokyo, Japan). Lipofectamin RNAiMAX (Invitrogen, Life Technologies, Carlsbad, CA) was used to transfect siRNAs into HK-2 cells (final concentration, 10 nM), according to the manufacturer’s protocol. The sense sequences of the siRNAs were as follows: NF-κB p65, 5'-AGAG-GACAUUGAGGUAUTT-3'; p21, 5'-CUUCGACUUUGUCAGTT-3'.}
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\text{Evaluation of cellular proliferation. Serum-starved HK-2 cells were incubated with or without indoxyl sulfate for 72 h, and then the}
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![Fig. 3. Expression of α-SMA and TGF-β1 induced by IS is repressed by NF-κB p65 small interfering RNA (siRNA) in HK-2 cells. A: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM) and then serum starved for 24 h. Cell lysates were immunoblotted using anti-p65 antibody. B: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM) and serum starved for 24 h, followed by IS (250 μM) for 48 h. Expression levels of α-SMA mRNA levels were measured by real-time PCR. C: experimental conditions were as described in B. Expression levels of TGF-β1 mRNA were measured by real-time PCR. D: experimental conditions were as described in B except treatment with IS for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-α-SMA and anti-TGF-β1 antibodies. E: experimental conditions were as described in B and C except cells were not transfected with NF-κB p65 siRNA. Expression levels of NF-κB p65 mRNA levels were measured by real-time PCR. Data are shown as means ± SE of three independent experiments for B, D, and E, and of four independent experiments for C. *P < 0.05 vs. untreated cells; #P < 0.05 vs. IS-treated cells.}
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cells were incubated with or without 5% FBS for 72 h. After culture, HK-2 cells were harvested by trypsin digestion and counted by Trypan blue staining.

Measurement of SA β-gal activity. Serum-starved cells incubated with or without indoxyl sulfate were fixed with 0.5% glutaraldehyde and then stained in 100 mM sodium phosphate (pH 6.0), containing 0.05% X-gal, 1 mM MgCl₂, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, and 0.1% Triton X-100 at 37°C for 12 h. SA β-gal-positive cells were counted in six randomly representative fields.

Quantitative real time PCR. Total RNA was isolated using Sepasol-RNA I Super (Nacalai Tesque). First-strand cDNAs were synthesized from template RNA (2 μg) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative real time PCR proceeded using Syber Premix Ex Taq II Green (Takara Bio, Shiga, Japan) and the LightCycler PCR system (Roche, Mannheim, Germany), according to the manufacturer’s protocol with the following oligonucleotide primers: p53, 5' -CCGCCAGTCAGATCCTAGCG-3' (forward) and 5' -AATCATCCATTGTGGGACG-3' (reverse); p21, 5' -GTGGACCTGTCACTGTCTT-3' (forward) and 5' -GCGTTTGGAGTGGTAGAAATCTG-3' (reverse); α-SMA, 5' -GTGGTGCCCCCTGAAAGCAT-3' (forward) and 5' -GCTGGGAAGTGAAGTCTCA-3' (reverse); TGF-β1, 5'-CAACAACTTCCTGGGATACCT-3' (forward) and 5'-CCACACTGCGCACAAAC-3' (reverse); GAPDH, 5'-ATGGGGAAGGTGGTCT-3' (forward) and 5'-GGGGTCATTGATGGCAACAT-3' (reverse). The expression of mRNA levels was measured as the ratio of each mRNA to GAPDH mRNA.

Immunoblotting. Immunoblotting was performed as described previously (38, 42). In brief, cell lysates were fractionated by SDS-PAGE on polyacrylamide gels, and proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). Phospho-p65 (Ser-276), p53, p21, TGF-β1, and α-SMA were detected using specific antibodies and normalized to α-tubulin. The protein bands were visualized using the enhanced Chemi-Lumi One system (Nacalai Tesque).

Transfection and luciferase assays. For measurement of NF-κB promoter activity, HK-2 cells were transfected with pNF-κB RE-TK hRluc (F) and a pGL3-SV40 plasmid using FuGENE HD (Roche.

**Fig. 4. Senescence of HK-2 cells by IS is inhibited by NF-κB inhibitors and NF-κB p65 siRNA.** A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min, followed by ISO (250 μM) for 72 h, and then senescence-associated β-galactosidase (SA β-gal) was stained, and the number of SA β-gal-positive cells was counted. B: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM) and serum starved for 24 h, followed by IS (250 μM) for 72 h. SA β-gal was then stained, and the number of SA β-gal-positive cells was counted. Data are means ± SE of four separate experiments for A and of three separate experiments for B. *P < 0.05 vs. untreated cells; #P < 0.05 vs. IS-treated cells.
Mannheim, Germany). After incubation for 24 h, the transfected HK-2 cells were incubated with or without indoxyl sulfate for 24 h. The renilla luciferase activity was measured and normalized to the firefly luciferase activity. For measurement of p53 promoter activity, HK-2 cells were transfected with pGL4-phTP53, expression plasmids (pCMV-MFlag-hsTP53 or pCMV_S-FLAG as a negative control), and a pRL-SV40 plasmid using FuGENE HD (Roche). After incubation for 24 h, the transfected HK-2 cells were incubated with or without indoxyl sulfate for 72 h. The firefly luciferase activity was measured and normalized to the renilla luciferase activity. The luciferase activity was measured with the Dual-Luciferase reporter assay system (Promega, Madison, WI), according to the manufacturer’s protocol, with a luminometer.

Design of animal experiments. Experimental rats were produced by the Kureha Corporation (2). We produced CRF in 7-wk-old male Sprague-Dawley rats (Clea, Tokyo, Japan) by 4/5-nephrectomy. Eleven weeks later, the rats were randomized to a control group with CRF or to a group with CRF that was treated with AST-120. AST-120 (4 g·kg⁻¹·day⁻¹) was administered to the rats with powder chow (CE-2, Clea) for 16 wk, and the control group received powder chow alone. One control CRF rat died of uremia at the 15th week. Data from normal and CRF rats were compared. Body weight, blood pressure, and serum and urine parameters of these rats have been described (2). The Animal Care Committee of Kureha Biomedical Research Laboratories approved the study, which proceeded according to the Guidelines for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society.

Histochemistry and immunohistochemistry. Paraffin-embedded sections of kidney tissues provided by Kureha Corporation from the described rat models (2) were deparaffinized and hydrated. For histochemistry, the sections were stained with MT to evaluate renal fibrosis. For immunohistochemistry, sections were stained according to the streptavidin-biotin complex method. Sections were incubated overnight with anti-phospho-p65 (Ser-276) (diluted 1:100), anti-p53 (diluted 1:50), anti-p21 (diluted 1:50), anti-β-gal (diluted 1:50), anti-TGF-β1 (diluted 1:50) and anti-α-SMA (diluted 1:100) antibodies to determine their localization in kidney tissues. Antigen was retrieved by microwave heating twice in 0.01 M citrate buffer (pH 6.0) for 5 min at 600 W. All sections were examined by light microscopy (DN100, E600, Nikon, Tokyo, Japan).

Statistical analysis. Results are expressed as means ± SE. Values between groups were compared using the analysis of variance and Fisher’s protected least significance difference test. Results were considered statistically significant at P < 0.05.

RESULTS

Indoxyl sulfate induces phosphorylation of NF-κB p65 on Ser-276 through ROS. We used 250 μM indoxyl sulfate, which is comparable to its mean serum level in patients on hemodialysis (32). We determined the effect of indoxyl sulfate on phosphorylation of NF-κB p65 p53 in 48 h. Expression levels of p53 mRNA levels were measured by real-time PCR.

A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min, followed by IS (250 μM) for 48 h. Expression levels of p53 mRNA were measured by real-time PCR. Data are means ± SE. Values of four separate experiments for A were compared in A except treatment with IS for 72 h was used in place of 48 h of serum starvation. Cell lysates were immunoblotted with NF-κB inhibitors and NF-κB p65 siRNA in HK-2 cells. A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min, followed by IS (250 μM) for 48 h. Expression levels of p53 mRNA were measured by real-time PCR.}

B: experimental conditions were as described in A except treatment with IS for 72 h was used in place of 48 h of cell starvation. Cell lysates were immunoblotted using anti-p53 antibody. C: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM) and serum starved for 24 h, followed by IS (250 μM) for 48 h. Expression levels of p53 mRNA were measured by real-time PCR. D: experimental conditions were as described in C except treatment with IS for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-p53 antibody. Data are means ± SE of four separate experiments for A and of three separate experiments for B, C, and D. *P < 0.05 vs. untreated cells; #P < 0.05 vs. IS-treated cells.

Fig. 5. Expression of p53 induced by IS is repressed by NF-κB inhibitors and NF-κB p65 siRNA in HK-2 cells. A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min, followed by IS (250 μM) for 48 h. Expression levels of p53 mRNA were measured by real-time PCR.
(Fig. 1B). Furthermore, we confirmed with an NF-κB promoter-reporter construct that indoxyl sulfate enhanced NF-κB activity, whereas NAC inhibited this effect (Fig. 1C). Thus indoxyl sulfate induces phosphorylation of NF-κB p65 through ROS in HK-2 cells, and the phosphorylation of NF-κB p65 correlates with NF-κB activity.

**Activation and induction of NF-κB by indoxyl sulfate promote expression of TGF-β1 and α-SMA.** Indoxyl sulfate activates NF-κB with subsequent expression of PAI-1 in HK-2 cells (28). However, the other cellular function of NF-κB induced by indoxyl sulfate remained unknown. We found that indoxyl sulfate induces expression of α-SMA in HK-2 cells (38) and promotes the progression of CRF accompanied by increased TGF-β1 expression in CRF rats (27). Inhibitors of NF-κB such as PDTC and isohelenin were used to determine whether NF-κB induced by indoxyl sulfate promotes the expression of α-SMA and TGF-β1 in HK-2 cells. PDTC and isohelenin block degradation of IκB and thereby inhibit NF-κB (23). PDTC and isohelenin significantly suppressed indoxyl sulfate-induced mRNA and protein expression of both α-SMA and TGF-β1 (Fig. 2, A–C). NF-κB p65 siRNA was used to examine the relationship between indoxyl sulfate-induced NF-κB and the expression of α-SMA and TGF-β1. The efficiency of knockdown of NF-κB p65 is shown in Fig. 3A. NF-κB p65 siRNA inhibited the indoxyl sulfate-induced mRNA and protein expression of α-SMA and TGF-β1 (Fig. 3, B–D). Furthermore, indoxyl sulfate promoted mRNA and protein expression of NF-κB p65 (Fig. 3, D and E). Thus indoxyl sulfate promotes the expression of α-SMA and TGF-β1 through activation and induction of NF-κB.

**Indoxyl sulfate accelerates cellular senescence through NF-κB.** We previously reported that NAC inhibits indoxyl sulfate-induced cellular senescence (38). In the present study, NAC suppressed the indoxyl sulfate-induced phosphorylation of NF-κB p65 (Fig. 1B). Therefore, we examined whether indoxyl sulfate-induced NF-κB accelerates HK-2 cell senescence. Inhibitors of NF-κB and NF-κB p65 siRNA significantly suppressed indoxyl sulfate-induced SA β-gal activity (Fig. 4, A and B). Therefore, indoxyl sulfate accelerates HK-2 cell senescence through activation and induction of NF-κB.

**Activation and induction of NF-κB by indoxyl sulfate promote expression of p53.** We further examined the effects of NF-κB inhibitors and NF-κB p65 siRNA on p53 expression induced by indoxyl sulfate, because p53 is a senescence marker gene and indoxyl sulfate promotes p53 expression through ROS production (38). Inhibitors of NF-κB and NF-κB p65 siRNA suppressed the indoxyl sulfate-induced mRNA and protein expression of p53 (Fig. 5, A–D). Thus activation and induction of NF-κB by indoxyl sulfate promote expression of p53 in HK-2 cells.

**Indoxyl sulfate-induced p53 expression triggers p53 accumulation in HK-2 cells.** We previously demonstrated that indoxyl sulfate induces the expression of p53 time-dependently in HK-2 cells (38). We hypothesized that indoxyl sulfate-induced p53 might regulate p53 expression. PFTα, a p53 inhibitor, suppressed indoxyl sulfate-induced mRNA and protein expression of p53 and p53 promoter activity in HK-2 cells (Fig. 6, A–C). Furthermore, p53 promoter activity was enhanced by overexpression of p53 as well as indoxyl sulfate (Fig. 6D). Therefore, activation and induc-
tion of NF-κB by indoxyl sulfate triggers p53 accumulation via p53 itself in HK-2 cells.

**Activation and induction of NF-κB by indoxyl sulfate regulate p21 expression.** We reported that indoxyl sulfate promotes expression of p21 (38), a senescence marker gene and a downstream molecule of p53. We examined the effects of NF-κB inhibitors and NF-κB p65 siRNA on indoxyl sulfate-induced p21 expression. NF-κB inhibitors suppressed the indoxyl sulfate-induced mRNA and protein expression of p21 (Fig. 7, A and B). However, NF-κB p65 siRNA promoted indoxyl sulfate-induced mRNA and protein expression of p21 (Fig. 7, C and D). Thus activation and induction of NF-κB by indoxyl sulfate regulate p21 expression in HK-2 cells.

**Activation and induction of NF-κB by indoxyl sulfate regulate cellular senescence through p21 expression.** To examine the function of p21, we synthesized siRNA specific to p21. The efficiency of knockdown of p21 is shown in Fig. 8A. Figure 8B as well as Fig. 3D shows that indoxyl sulfate upregulated expression of NF-κB p65. NF-κB p65 siRNA promoted indoxyl sulfate-induced p21 expression, whereas NF-κB p65/p21 siRNAs abrogated p21 expression (Fig. 8B). Then we evaluated the effect of indoxyl sulfate on cellular senescence. NF-κB p65 siRNA inhibited the number of SA-β-gal-positive cells in the presence of indoxyl sulfate, although p21 was upregulated (Fig. 8, C and D). Furthermore, NF-κB p65/p21 siRNAs more intensely inhibited the number of SA-β-gal-positive cells in the presence of indoxyl sulfate than NF-κB p65 siRNA alone. Therefore, indoxyl sulfate-induced cellular senescence is regulated by expression of NF-κB p65 and p21 in HK-2 cells.

**Activation and induction of NF-κB by indoxyl sulfate regulate cellular proliferation.** Accumulating evidence demonstrates that both p53 and p21 induce cellular senescence and suppress cellular proliferation. Indoxyl sulfate suppressed serum-induced cellular proliferation of HK-2 cells (Fig. 9, A and B). NF-κB inhibitors alleviated indoxyl sulfate-induced suppression of proliferation (Fig. 9A). NF-κB p65 siRNA-transfected cells showed less cellular proliferation in the presence of indoxyl sulfate than that in control cells (Fig. 9B). This result might be due to indoxyl sulfate-promoted p21 expression by knockdown of NF-κB p65. NF-κB p65/p21 siRNAs-transfected cells showed less cellular proliferation in the presence of indoxyl sulfate than that in NF-κB p65 siRNA-transfected cells. Thus NF-κB p65 and p21 cooperatively regulate indoxyl sulfate-induced suppression of cellular proliferation.

**Phosphorylated NF-κB p65, p53, p21, β-gal, TGF-β1, and α-SMA are colocalized in renal tubules of CRF rats.** AST-120 suppressed accumulation of indoxyl sulfate in the serum and renal tubules of CRF rats (25). Furthermore, AST-120 suppressed ROS production (31, 40), expression of TGF-β-SMA are colocalized in renal tubules of CRF rats.

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**Fig. 7.** Expression of p21 induced by IS is repressed by NF-κB inhibitors and NF-κB p65 siRNA in HK-2 cells. A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min, followed by IS (250 μM) for 48 h. Expression levels of p21 mRNA levels were measured by real-time PCR. B: experimental conditions were as described in A except treatment with IS for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-p21 antibody. C: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM) and serum starved for 24 h, followed by IS (250 μM) for 48 h. Expression levels of p21 mRNA levels were measured by real-time PCR. D: experimental conditions were as described in C except treatment with IS for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-p21 antibody. Data are means ± SE of three separate experiments for A, B, C, and D. *P < 0.05 vs. untreated cells; #P < 0.05 vs. IS-treated cells.
p53, α-SMA (38), and SA β-gal (2, 38) in the renal tubules of CRF rats. To determine whether phosphorylated NF-κB p65 is colocalized with p53, p21, β-gal, TGF-β1, and α-SMA, we examined renal cortex sections from CRF rats. Figure 10A shows that phosphorylated NF-κB p65, p53, p21, β-gal, TGF-β1, and α-SMA were colocalized in the renal tubules. CRF rats showed more intense tubular staining of phosphorylated NF-κB p65, p53, p21, β-gal, TGF-β1, and α-SMA, and MT-positive fibrosis area in the kidneys than normal and AST-120-treated CRF rats (Fig. 10, A and B).

DISCUSSION

The present findings suggest the following mechanisms (Fig. 11). Indoxyl sulfate promotes activation (phosphorylation) of NF-κB p65 through ROS, followed by p53 expression. Indoxyl sulfate-induced p53 expression triggers p53 accumulation. Activation and induction of NF-κB by indoxyl sulfate suppress cellular proliferation and induce cellular senescence and expression of TGF-β1 and α-SMA, accompanied by renal fibrosis. AST-120, which removes indoxyl sulfate, inhibits tubular expression of phosphorylated NF-κB p65, p53, p21, β-gal, TGF-β1, and α-SMA and MT-positive fibrosis area in the kidneys of CRF rats. Taken together, NF-κB plays an important role in indoxyl sulfate-induced cellular senescence, fibrotic gene expression, and inhibition of proliferation in proximal tubular cells. More notably, indoxyl sulfate accelerates proximal tubular cellular senescence with progression of CRF through the ROS-NF-κB-p53 pathway.

We found that NF-κB activation by indoxyl sulfate regulates the senescence of proximal tubular cells. Studies in vitro using NF-κB knockout mouse embryonic fibroblasts demonstrated that NF-κB induces cellular senescence (43). The NF-κB pathway is a candidate activator of age-related transcriptional changes in human and mouse tissues (3, 4, 7, 16), and NF-κB is activated particularly in the glomeruli of aging kidneys (45). Thus indoxyl sulfate-induced activation of NF-κB accelerates the senescence of proximal tubular cells.

Indoxyl sulfate induced expression of NF-κB p65 (Figs. 3, D and E, and 8B). In the promoter region of NF-κB p65, one NF-κB p65 binding site exists (chr11: 65421932–65421942; TGGGGAGCCCC). Therefore, NF-κB p65 expression might be associated with indoxyl sulfate-induced activation of NF-κB p65. On the other hand, Rajani Ravi et al. (35) reported that p53 suppresses expression of NF-κB p65. In the present study, indoxyl sulfate upregulates NF-κB p65 (Figs. 3, D and E, and 8B), although p53 expression is promoted by indoxyl sulfate. We consider that p53 does not
effectively regulate NF-κB p65 expression in indoxyl sulfate-treated HK-2 cells.

NF-κB inhibitors and NF-κB p65 siRNA suppressed indoxyl sulfate-induced TGF-β1 and α-SMA expression (Figs. 2, A–C and 3, B–D). NF-κB activation directly regulates TGF-β1 expression in many cell types. Therefore, indoxyl sulfate-induced activation of NF-κB might directly regulate TGF-β1 expression in proximal tubular cells. On the contrary, there is no NF-κB p65 binding site in human α-SMA promoter region. Indoxyl sulfate-induced p53 expression through NF-κB activation might regulate α-SMA expression, because expression of p53 is increased by indoxyl sulfate-induced NF-κB activation (Fig. 5, A–D), and p53 can bind to the α-SMA promoter region and directly regulate α-SMA expression in the human osteogenic sarcoma cell line Saos-2 (8).

Indoxyl sulfate accelerated expression of α-SMA in proximal tubular cells and thereby renal fibrosis. TGF-β1 also induces this effect (11). Indoxyl sulfate induced TGF-β1 expression in proximal tubular cells (Fig. 3, C and D). Indoxyl sulfate and secreted TGF-β1 induced by indoxyl sulfate might additively or synergistically promote expression of α-SMA in proximal tubular cells. Therefore, the cross talk between indoxyl sulfate and TGF-β1 should be investigated. As a possible cross talk, indoxyl sulfate downregulates Klotho expression in proximal tubular cells and rat kidneys (37). Klotho protein directly binds to the type-II TGF-β receptor and suppresses TGF-β1 signaling, thereby inhibiting renal fibrosis (9). Thus suppression of Klotho expression by indoxyl sulfate might be involved in the enhancement of TGF-β1 signaling.

Expression of p53 induced by indoxyl sulfate was activated by NF-κB in proximal tubular cells (Fig. 5, A–D). This finding is consistent with the other reports that NF-κB activation involves p53 expression (5, 6, 17, 20, 34). NF-κB directly binds to p53 promoter region and thereby controls p53 expression. Furthermore, the p53 promoter contains p53 binding sites. NF-κB subunits and p53 additively affect activation of the p53 promoter (6). In addition, p53 directly regulates p53 transcription (44). The present study showed that PFTα inhibits indoxyl sulfate-promoted expression of p53 and p53 promoter activity (Fig. 6, A–D). Moreover, overexpression of p53 increases p53 promoter activity (Fig. 6D). Taken together, indoxyl sulfate-induced expression of p53 might be directly regulated by not only the activation of NF-κB but also p53 itself in proximal tubular cells. Thus indoxyl sulfate-induced activation of NF-κB triggers p53 accumulation via the induction of p53 itself.

Indoxyl sulfate-induced p21 mRNA expression was suppressed by NF-κB inhibitors, whereas NF-κB p65 siRNA promoted indoxyl sulfate-induced p21 expression. We consider that the effects of NF-κB inhibitors were not artifacts, because two kinds of inhibitors produced the same results (Fig. 7, A and B). Thus NF-κB highly influences indoxyl sulfate-induced p21 expression. Indoxyl sulfate-induced mRNA expression of p21 might be regulated by at least two mechanisms. As one mechanism, p21 expression is regulated by p53 (38), which is promoted by indoxyl sulfate-induced NF-κB activation and induction. As the second mechanism, p21 expression is interrupted by indoxyl sulfate-induced activation of NF-κB partially through its binding to the other transcription factors or p21 promoter region. For instance, cotransfection of NF-κB p65 and peroxisome proliferator-activated receptor-γ (PPAR-γ) suppressed PPAR-γ-induced phosphoenolpyruvate carboxykinase (PEPCK) promoter activation (36). In addition, Hwang et al. reported that NF-κB suppresses expression of cyclin D1 through binding to cyclin D1 promoter (18). Therefore, similar mechanisms might exist in the pathways of indoxyl sulfate–induced p21 expression because the other transcription factors except p53 regulate p21 expression and the promoter region of p21 contains NF-κB binding sites.

p21 is a main regulatory factor in cellular senescence. In proximal tubular cells, aldosterone induces cellular senescence via p21-dependent pathway (14). However, NF-κB p65 siRNA suppresses indoxyl sulfate-induced SA-β-gal activity, although p21 is highly expressed. Thus activation of NF-κB p65 might be more important in indoxyl sulfate-induced senescence of HK-2 cells than p21 expression.

NF-κB p65 siRNA-transfected cells showed less proliferation in the presence of indoxyl sulfate than control cells (Fig. 9B). Indoxyl sulfate induced p21 expression more intensely in

Fig. 9. Suppression of serum-induced proliferation of HK-2 cells by IS was regulated by NF-κB. A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min followed by IS (250 μM) for 72 h. Cells were incubated with or without 5% FBS for 72 h, and then proliferation was assayed. B: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM) and p21 siRNA (10 nM) and serum starved for 24 h, followed by IS (250 μM) for 72 h. Cells were incubated with or without 5% FBS for 72 h, and then proliferation was assayed. Data are shown as means ± SE of four separate experiments for A and of three separate experiments for B. *P < 0.05 vs. untreated cells; #P < 0.05 vs. IS-treated cells.
NF-κB p65 siRNA-transfected cells than that in control cells (Figs. 7, C and D, and 8B). Taken together, we predicted that indoxyl sulfate-induced expression of p21 with NF-κB p65 siRNA suppressed cellular proliferation. However, NF-κB p65/p21 siRNAs-transfected cells showed less proliferation in the presence of indoxyl sulfate than NF-κB p65 siRNA-transfected cells (Fig. 9B). This result might be due to disruption of cell cycle regulatory gene expression. For instance, a loss of p21 augments p14 Arf expression in human carcinoma cells (15). Figure 9A also supports the hypothesis that expression of cell cycle regulatory genes might be modulated because NF-κB inhibitors partially alleviated indoxyl sulfate-induced inhibition of serum-dependent cellular proliferation even in the downregulation of p53 and p21 expression. However, further study would be required to clarify the mechanistic details.

The present results were obtained from cells and animal experiments. A clinical study is required to prove that the activation of NF-κB is involved in cell senescence, expression of TGF-β1, and fibrosis in the kidneys of patients with CRF. This might be useful for developing medicines against NF-κB to treat CRF patients.
Furthermore, the clinical effects of AST-120 on NF-κB activation in the kidney should also be studied because AST-120 suppresses CRF progression. Activation of NF-κB by indoxyl sulfate is accompanied by the suppression of cell proliferation and the initiation of fibrosis and senescence in proximal tubular cells. Understanding the process of NF-κB activation by indoxyl sulfate is necessary to clarify the molecular mechanisms of indoxyl sulfate actions not only in proximal tubular cells but also in the other types of cells such as vascular smooth muscle cells and endothelial cells. AST-120 that can reduce serum indoxyl sulfate suppressed expression of phosphorylated NF-κB p65, p53, p21, β-gal, TGF-β1, and α-SMA in the kidneys of CRF rats. Furthermore, a diet rich in antioxidants might attenuate the actions of indoxyl sulfate as AST-120 does, because NF-κB is a redox-dependent transcription factor.

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DISCLOSURES

F. Nishijima is employed by Kureha Corporation. The other authors declare no competing interests.

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