CREB, NF-κB, and NADPH oxidase coordinately upregulate indoxyl sulfate-induced angiotensinogen expression in proximal tubular cells

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UREMIC TOXINS ACCUMULATE IN serum when renal function deteriorates. Especially, indoxyl sulfate, a representative uremic toxin, induces nephrotoxicity. Administration of indoxyl sulfate and its precursors stimulates the progression of glomerular sclerosis as well as tubulointerstitial fibrosis (33, 34). Indoxyl sulfate increased the expression of transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloprotease-1, and pro-α1(I) collagen in uremic rat kidneys (27, 28). Indoxyl sulfate increases the expression of transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloprotease-1, and pro-α1(I) collagen in uremic rat kidneys (27, 28). Indoxyl sulfate increases the expression of transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloprotease-1, and pro-α1(I) collagen in uremic rat kidneys (27, 28). Indoxyl sulfate increases the expression of transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloprotease-1, and pro-α1(I) collagen in uremic rat kidneys (27, 28).

The present study aimed to determine the relationship between indoxyl sulfate and the upregulation of AGT expression in proximal tubular cells. Indoxyl sulfate induced expression of AGT in rat renal cortex and in cultured human proximal tubular cells (1, 9, 13, 36, 57, 58). The present study aimed to determine the relationship between indoxyl sulfate and the upregulation of AGT expression in proximal tubular cells. Indoxyl sulfate induced expression of AGT in rat renal cortex and in cultured human proximal tubular cells (1, 9, 13, 36, 57, 58).

MATERIALS AND METHODS

Reagents. Antibodies were obtained from the following suppliers: anti-α-tubulin for immunoblotting, Calbiochem (La Jolla, CA); anti-phospho-CREB (Ser-133), anti-CREB, and anti-p53 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); and anti-NADPH oxidase 4 (NOX4) for immunoblotting, Abcam (Cambridge, UK). Indoxyl sulfate was from Alfa Aesar (Lancashire, UK). Pyrrolidine dithiocarbamate (PDTC), isohelenin (ISO), diphenyleneiodonium chloride (DPI), and N-acetylcysteine (NAC) were purchased from Nacalai Tesque (Kyoto, Japan). Trypsin-EDTA, FBS, and insulin-transferrin-serum were purchased from GIBCO (Grand Island, NY). Penicillin and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan).

CELL CULTURE. HK-2 cells derived from human proximal tubular cells were purchased from ATCC (Manassas, VA) and were maintained in a medium containing 10% FBS.
DMEM/F12 supplemented with 10% FBS, insulin-transferrin- selenium, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of small interfering RNAs specific to CREB and NF-κB p65. Small interfering RNAs (siRNAs) specific to CREB and NF-κB p65 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: CREB, 5'-GAGAGGAGGCUGUCUAUAGdTDtT-3'; and NF-κB p65, 5'-AGAGGACAUUGGGUGAUUAUdTdT-3'.

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 was performed with Super 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: rat AGT, 5'-GTTAGGAGTCCTCGCTTCTCCA-3' (forward) and 5'-GTGGTGAGGATCCCGAATTTC-3' (reverse); rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CTGGTGTCTGAGGCT-3' (forward) and 5'-CTGTGTTGCTGAGGATCCCGAATTTC-3' (reverse); human AGT, 5'-CTGGTGTCTGAGGCT-3' (forward) and 5'-CTGTGTTGCTGAGGATCCCGAATTTC-3' (reverse); human CREB, 5'-CCACGTGTAACGGCTGGAACACT-3' (forward) and 5'-GCTGATTTGCTCA-TGGTAAATGT-3' (reverse); human NF-κB p65, 5'-TTGGCTTACCTGGAAGTGTTGAATTGACAGG-3' (forward) and 5'-GCACATCACGGTGC-GAAAGG-3' (reverse); human GAPDH, 5'-GCAAAGCTGAGACTCA-GATC-3' (reverse) and 5'-GCAAAGCTGAGACTCA-GATC-3' (forward); human NOX4, 5'-CCGCTTGCATCAGTCTTAAACC-3' (forward) and 5'-TCGGCAGATCAAGGCAAAAC-3' (reverse); and human GAPDH, 5'-ATGGGAGGTGGAAGGTGAGG-3' (forward) and 5'-GGGGTCATTGATGGCAACCTTGC-3' (reverse).

Immunoblotting. Immunoblotting was performed as described previously (45, 47). In brief, cell lysates were fractionated by SDS-PAGE on polyacrylamide gels, and proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA). Phospho-CREB (Ser-133), CREB, NF-κB p65, and NOX4 were detected using specific antibodies and were normalized to α-tubulin. The protein bands were visualized using the enhanced Chemi-Lumi One system (Nacalai Tesque).

Design of experimental rats. Experimental rats were produced by Kureha as reported previously (2, 3, 7). Briefly, 5-wk-old Dahl salt-resistant rats (Dahl-Iwai, n = 16) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and were fed powder rat chow (CE-2; Clea, Tokyo, Japan) and water. At 16 wk of age, the rats were divided into two groups: 1) Dahl salt-resistant normotensive rats (DN), and 2) Dahl salt-resistant normotensive indoxyl sulfate-administered rats (DN + IS; 200 mg kg⁻¹ day⁻¹ of indoxyl sulfate in drinking water). After 32 wk, the rats were anesthetized, and then renal cortices were isolated. This experiment was approved by the Animal Care Committee of Biomedical Research Laboratories of Kureha and proceeded according to the Guiding Principles for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society.

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

RESULTS

AGT is upregulated in the renal cortex of indoxyl sulfate-administered rats and in indoxyl sulfate-stimulated proximal tubular cells. We prepared indoxyl sulfate-administered rats (2, 3, 7). At the 32nd wk of the study, serum indoxyl sulfate levels were 0.10 ± 0.01 mg/dl in control rats and 0.94 ± 0.13 mg/dl in indoxyl sulfate-administered rats (P < 0.001 vs. control). Serum levels of indoxyl sulfate in indoxyl sulfate-administered rats were rather below the mean serum level of indoxyl sulfate (5.3 mg/dl) in patients on hemodialysis (33). Systolic blood pressure levels at the 32nd wk of the study were 143 ± 3 mmHg in control rats and 141 ± 3 mmHg in indoxyl sulfate-administered rats. Urinary protein levels at the 32nd wk of the study were 95.8 ± 23.5 mg/day in control rats and 99.2 ± 21.8 mg/day in indoxyl sulfate-administered rats. The expression level of AGT was increased in renal cortex of indoxyl sulfate-administered rats compared with control rats (Fig. 1A).

For in vitro experiments, indoxyl sulfate at a concentration of 250 μM, which is comparable to its mean serum level in patients on hemodialysis (33) was used. Indoxyl sulfate time dependently increased the expression of AGT in HK-2 cells (Fig. 1B). Thus indoxyl sulfate induced the expression of AGT in rat kidneys as well as in human proximal tubular cells.

CREB promotes indoxyl sulfate-induced AGT expression in proximal tubular cells. Accumulating evidence indicates that CREB directly regulates AGT expression in proximal tubular cells (9, 36, 57, 58). Indoxyl sulfate time dependently induced phosphorylation of CREB on Ser-133 (Fig. 2A), which is necessary for its binding to CRE sequence of the specific gene promoter and therefore initiates gene transcription (38, 56). To examine the effect of CREB on indoxyl sulfate-induced AGT expression, CREB siRNA was prepared (Fig. 2B). The expres-

Fig. 1. Indoxyl sulfate induces angiotensinogen (AGT) expression in the renal cortex of indoxyl sulfate (IS)-administered rats and in indoxyl sulfate-stimulated proximal tubular cells. A: expression levels of AGT were measured by real-time PCR. Data are expressed as means ± SE (control rats, n = 8; indoxyl sulfate-administered rats, n = 8). *P < 0.05 vs. control. B: serum-starved HK-2 cells were incubated with indoxyl sulfate (250 μM) for indicated periods. Expression levels of AGT mRNA were measured by real-time PCR. Data are expressed as means ± SE of 5 independent experiments. *P < 0.05 vs. untreated cells.
Fig. 2. Indoxyl sulfate induces AGT expression through cAMP response element-binding protein (CREB) phosphorylation in proximal tubular cells. A: serum-starved HK-2 cells were incubated with or without indoxyl sulfate (250 μM) for 24 h, and then cell lysates were immunoblotted using anti-phospho-CREB (Ser-133) antibody. B: HK-2 cells were transfected with or without CREB small interfering (si)RNA (10 nM) and then serum starved for 48 h. Cell lysates were immunoblotted using anti-CREB antibody. C: HK-2 cells were transfected with or without CREB siRNA (10 nM), and serum starved for 48 h, followed by incubation with indoxyl sulfate (250 μM) for 48 h. Expression levels of AGT mRNA were measured by real-time PCR. D: experimental conditions were as described in C except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-AGT antibody. Data are shown as means ± SE of 4 independent experiments for C. *P < 0.05 vs. untreated cells; #P < 0.05 vs. indoxyl sulfate alone.

Fig. 3. NF-κB promotes indoxyl sulfate-induced AGT expression in proximal tubular cells. Active NF-κB directly binds and activates the AGT promoter in proximal tubular cells (1). Indoxyl sulfate activates NF-κB in HK-2 cells (29, 45). Therefore, we next checked whether NF-κB regulates indoxyl sulfate-induced AGT expression. NF-κB inhibitors, PDTC and ISO, suppressed indoxyl sulfate-induced expression of AGT mRNA and protein (Fig. 3, A and B). Furthermore, when NF-κB p65 expression was knocked down by NF-κB p65 siRNA (Fig. 3C), indoxyl sulfate-induced expression of AGT mRNA and protein was suppressed (Fig. 3, D and E). Thus NF-κB promotes indoxyl sulfate-induced AGT expression in proximal tubular cells.

NF-κB promotes indoxyl sulfate-induced CREB expression in proximal tubular cells. Because expression of CREB protein is upregulated by indoxyl sulfate (Fig. 2D) and NF-κB regulates CREB expression (8), we examined whether indoxyl sulfate-induced NF-κB activation promotes CREB expression in HK-2 cells. Both PDTC and ISO inhibited indoxyl sulfate-induced expression of CREB mRNA and protein (Fig. 4, A and B). NF-κB p65 siRNA also suppressed indoxyl sulfate-induced expression of CREB mRNA and protein (Fig. 4, C and D). Therefore, NF-κB promotes indoxyl sulfate-induced CREB expression in HK-2 cells.

ROS promotes indoxyl sulfate-induced CREB and NF-κB p65 expression in proximal tubular cells. Because indoxyl sulfate-induced ROS production activates NF-κB (29, 45), we examined the relationship between ROS and CREB expression. NAC, an antioxidant, inhibited indoxyl sulfate-induced expression of CREB mRNA and protein (Fig. 5, A and B). We also examined whether NADPH oxidase regulates indoxyl sulfate-induced CREB expression. DPI, an NADPH oxidase inhibitor, suppressed indoxyl sulfate-induced expression of CREB mRNA and protein (Fig. 5, C and D).

Furthermore, we determined the relationship between ROS and NF-κB p65 expression. Both NAC and DPI suppressed indoxyl sulfate-induced expression of NF-κB p65 mRNA and protein (Fig. 6, A–D). Thus NADPH oxidase-induced ROS production upregulates indoxyl sulfate-stimulated expression of CREB and NF-κB p65 in proximal tubular cells.

CREB promotes indoxyl sulfate-induced NF-κB p65 expression in proximal tubular cells. In the present study, NF-κB promoted indoxyl sulfate-induced CREB expression (Fig. 4).
Therefore, we examined whether CREB promotes indoxyl sulfate-induced NF-κB p65 expression. CREB siRNA inhibited indoxyl sulfate-induced expression of NF-κB p65 mRNA and protein (Fig. 7, A and B). Thus CREB promotes indoxyl sulfate-induced NF-κB p65 expression in proximal tubular cells.

**ROS promotes indoxyl sulfate-induced AGT expression in proximal tubular cells.** Production of ROS upregulated indoxyl sulfate-induced NF-κB p65 and CREB expression (Figs. 5 and 6). Therefore, we predicted that ROS production upregulates indoxyl sulfate-induced AGT expression. Both NAC and DPI inhibited indoxyl sulfate-induced expression of AGT mRNA and protein (Fig. 8, A–D). Thus indoxyl sulfate-induced ROS production upregulates AGT expression in proximal tubular cells.

Both NF-κB p65 and CREB promote indoxyl sulfate-induced NOX4 expression in proximal tubular cells. We previously reported that indoxyl sulfate induces the expression of NOX4, an NADPH oxidase, in vascular smooth muscle cells and vascular endothelial cells (30, 54). Therefore, we examined whether NF-κB and CREB promote indoxyl sulfate-induced NOX4 expression. Indoxyl sulfate induced expression of NOX4 mRNA, although NOX4 mRNA was not detected in untreated cells (data not shown). Indoxyl sulfate induced expression of NOX4 protein, whereas NF-κB inhibitors (PDTC and ISO), NF-κB p65 siRNA, and CREB siRNA suppressed indoxyl sulfate-induced expression of NOX4 protein (Fig. 9, A–C). Thus both NF-κB and CREB upregulate indoxyl sulfate-induced NOX4 expression in proximal tubular cells.

**ROS promotes indoxyl sulfate-induced AGT and NOX4 expression in proximal tubular cells.** We determined whether ROS production upregulates indoxyl sulfate-induced NOX4 expression. Both NAC and DPI inhibited indoxyl sulfate-induced NOX4 expression (Fig. 10, A and B). Thus indoxyl sulfate-induced ROS production upregulates NOX4 expression in proximal tubular cells.

**DISCUSSION**

The novel findings in the present study are as follows: 1) ROS, NF-κB, and CREB upregulate indoxyl sulfate-induced AGT expression in proximal tubular cells; 2) ROS promotes indoxyl sulfate-induced NF-κB p65 and CREB expression; 3) NF-κB p65 and CREB expression upregulated by indoxyl sulfate regulate each other; and 4) ROS, NF-κB, and CREB promote indoxyl sulfate-induced NOX4 expression. Taken together, NF-κB, CREB, and p65 promote AGT expression and NOX4 expression, which are major contributors to proteinuria.

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**Figure Captions**

**Fig. 4.** NF-κB promotes indoxyl sulfate-induced CREB expression in proximal tubular cells. A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of CREB mRNA were measured by real-time PCR. B: experimental conditions were as described in A except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-CREB antibody. C: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM), and serum starved for 24 h, followed by incubation with indoxyl sulfate (250 μM) for 48 h. Expression levels of CREB mRNA were measured by real-time PCR. D: experimental conditions were as described in C except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-CREB antibody. Data are shown as means ± SE of 4 independent experiments for A and C; *P < 0.05 vs. untreated cells; #P < 0.05 vs. indoxyl sulfate alone.

**Fig. 5.** Reactive oxygen species (ROS) promotes indoxyl sulfate-induced CREB expression in proximal tubular cells. A: serum-starved HK-2 cells were incubated with or without N-acetylcyesteine (NAC; 10 mM) for 30 min, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of CREB mRNA were measured by real-time PCR. B: experimental conditions were as described in A except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-CREB antibody. C: serum-starved HK-2 cells were incubated with or without diphenyleneiodonium (DPI; 10 μM) for 30 min, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of CREB mRNA were measured by real-time PCR. D: experimental conditions were as described in C except for treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-CREB antibody. Data are shown as means ± SE of 4 independent experiments for A and of 4 independent experiments for C. *P < 0.05 vs. untreated cells; #P < 0.05 vs. indoxyl sulfate alone.
CREB, and NOX4 coordinately regulate each other and play an important role in indoxyl sulfate-induced AGT expression in proximal tubular cells.

AGT expression is upregulated by angiotensin II in vitro and in vivo (15, 19, 20, 40, 51). When AGT is upregulated, angiotensin II is also increased because AGT is a precursor of angiotensin II. As demonstrated in the present study, indoxyl sulfate induces AGT expression. Thus indoxyl sulfate might be a trigger of a positive feedback loop of angiotensin II-AGT. Furthermore, indoxyl sulfate-induced expression of AGT might be enhanced by angiotensin II and interleukin-6 through indoxyl sulfate-promoted expression of NF-κB p65 and CREB, because activation of NF-κB and CREB is involved in AGT expression by angiotensin II and interleukin-6 in proximal tubular cells (1, 8, 36, 57, 58).

A recent study has called into question the origin of proximal tubular AGT, indicating that liver-derived AGT is the primary source of renal AGT protein especially in conditions of damage of the glomerular basement membrane (26). However, the animal model used in this study was not associated with advanced renal dysfunction. Our present study suggests that indoxyl sulfate accumulated in serum of advanced renal dysfunction induces AGT expression in proximal tubular cells. Thus the expression mechanism of AGT in proximal tubules might be different between early and advanced stage of CKD depending on the degree of renal dysfunction and consequent serum indoxyl sulfate level.

Indoxyl sulfate administration did not make rats hypertensive. Increased local expression of AGT in proximal tubular cells might not be enough to affect systemic blood pressure. However, it is possible that further administration of indoxyl sulfate might induce hypertension in the rats. There was no significant difference in kidney fibrosis in normotensive rats treated with indoxyl sulfate (7). However, indoxyl sulfate administration induced early signs of kidney fibrosis such as upregulation of TGF-β1, monocyte chemotactic protein-1, α-SMA, and intercellular adhesion molecule-1 in the kidney of the normotensive rats (7, 41, 42, 44). Further administration of indoxyl sulfate might induce kidney fibrosis even in normotensive rats. In fact, indoxyl sulfate administration induced kidney fibrosis in hypertensive rats (7).

CREB activates OAT3 promoter and induces expression of OAT3 (35). We previously reported that indoxyl sulfate increases OAT3 expression in the kidneys of indoxyl sulfate-administered CKD rats (10). Therefore, indoxyl sulfate-induced OAT3 expression might be promoted by CREB in proximal tubular cells. Furthermore, activation of OAT3 is induced by epidermal growth factor (EGF; Ref. 49), and EGF receptor is activated by angiotensin II in the kidney (24). Taken together, indoxyl sulfate-induced expression of OAT3 and AGT might enhance uptake of indoxyl sulfate by proximal tubular cells.

Indoxyl sulfate induces NOX4 expression in proximal tubular cells. ROS production is increased in the kidney of CKD rats (31, 50). NOX4-induced ROS production by indoxyl

Fig. 6. ROS promotes indoxyl sulfate-induced NF-κB p65 expression in proximal tubular cells. A: serum-starved HK-2 cells were incubated with or without NAC (10 mM) for 30 min, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of NF-κB p65 mRNA were measured by real-time PCR. B: experimental conditions were as described in A except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-NF-κB p65 antibody. C: serum-starved HK-2 cells were incubated with or without DPI (10 μM) for 30 min, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of NF-κB p65 mRNA were measured by real-time PCR. D: experimental conditions were as described in C except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-NF-κB p65 antibody. Data are shown as means ± SE of 3 independent experiments for A and of 5 independent experiments for C. *P < 0.05 vs. untreated cells; #P < 0.05 vs. indoxyl sulfate alone.

Fig. 7. CREB promotes indoxyl sulfate-induced NF-κB p65 expression in proximal tubular cells. A: HK-2 cells were transfected with or without CREB siRNA (10 nM), and serum starved for 48 h, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of NF-κB p65 mRNA levels were measured by real-time PCR. B: experimental conditions were as described in C except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-NF-κB p65 antibody. Data are shown as means ± SE of 4 independent experiments for A. *P < 0.05 vs. untreated cells; #P < 0.05 vs. indoxyl sulfate alone.
sulfate might attenuate activity of protein tyrosine phosphatases (PTPs) in the kidney, because NOX4 constitutively generates ROS (11), and ROS suppresses activity of PTPs (52, 53). Therefore, phosphotyrosine proteins might be increased in indoxyl sulfate-stimulated proximal tubular cells and in the kidney of indoxyl sulfate-administered rats. In fact, tyrosine of Stat3 is phosphorylated in the kidney of CKD rats (43), although some PTPs might be able to dephosphorylate phosphotyrosine of Stat3 (12, 59). Stat3 promotes indoxyl sulfate-induced NOX4 expression, because Stat3 is involved in upregulation of AGT expression in proximal tubular cells (39). Taken together, constitutive production of ROS by NOX4 might induce dysfunction and senescence of proximal tubular cells through upregulation of phosphotyrosine proteins such as Stat3 by inactivation of PTPs.

An oral absorbent AST-120 is clinically used in Japan to delay CKD progression and dialysis initiation (22, 23, 25, 48, 55). AST-120 suppresses accumulation of indoxyl sulfate in the serum and renal tubules by removing its precursor, indole, from the intestine and thereby prevents kidney fibrosis and dysfunction (27, 28, 45, 47). Combination therapy with a RAS blocker and AST-120 might attenuate CKD progression more effectively than a RAS blocker alone, because AGT expression is upregulated by indoxyl sulfate as well as angiotensin II (14, 15, 19, 20, 40, 51). In uremic rats, a combination therapy with an ACE inhibitor and AST-120 was more effective than an ACE inhibitor alone in slowing the progression of CKD by inhibiting glomerular sclerosis and interstitial fibrosis (6, 37). Combination of AST-120 with a low-protein diet and a RAS blocker delayed the deterioration of CKD in patients with early CKD.

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**Fig. 8.** ROS promotes indoxyl sulfate-induced AGT expression in proximal tubular cells. A: serum-starved HK-2 cells were incubated with or without NAC (10 mM) for 30 min, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of AGT were measured by real-time PCR. B: experimental conditions were as described in A except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-AGT antibody. C: serum-starved HK-2 cells were incubated with or without DPI (10 μM) for 30 min, followed by indoxyl sulfate (250 μM) for 48 h. Expression levels of AGT mRNA were measured by real-time PCR. D: experimental conditions were as described in C except treatment with indoxyl sulfate for 72 h was used in place of 48 h. Cell lysates were immunoblotted using anti-AGT antibody. Data are shown as means ± SE of 4 independent experiments for A and C. *P < 0.05 vs. untreated cells; #P < 0.05 vs. indoxyl sulfate alone.

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**Fig. 9.** NF-κB and CREB promotes indoxyl sulfate-induced NADPH oxidase 4 (NOX4) expression in proximal tubular cells. A: serum-starved HK-2 cells were incubated with or without PDTC (10 μM) or ISO (10 μM) for 30 min, followed by indoxyl sulfate (250 μM) for 72 h. Cell lysates were immunoblotted using anti-NOX4 antibody. B: HK-2 cells were transfected with or without NF-κB p65 siRNA (10 nM), and serum starved for 24 h, followed by incubation with indoxyl sulfate (250 μM) for 72 h. Cell lysates were immunoblotted using anti-NOX4 antibody. C: HK-2 cells were transfected with or without CREB siRNA (10 nM) and serum starved for 48 h, followed by indoxyl sulfate (250 μM) for 72 h. Cell lysates were immunoblotted using anti-NOX4 antibody.

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**Fig. 10.** ROS promotes indoxyl sulfate-induced NOX4 expression in proximal tubular cells. A: serum-starved HK-2 cells were incubated with or without NAC (10 mM) for 30 min, followed by indoxyl sulfate (250 μM) for 72 h. Cell lysates were immunoblotted using anti-NOX4 antibody. B: serum-starved HK-2 cells were incubated with or without DPI (10 μM) for 30 min, followed by indoxyl sulfate (250 μM) for 72 h. Cell lysates were immunoblotted using anti-NOX4 antibody.
or rapid progression compared with a low-protein diet and a RAS blocker (60). Further clinical studies would be required to clarify the effect of the combination therapy with a RAS blocker and AST-120 on the progression of CKD.

**DISCLOSURES**

Y. Higashiyama and F. Nishijima are employed by Kureha. The other authors declare no competing interests.

**AUTHOR CONTRIBUTIONS**

Author contributions: H.S. and T.N. conception and design of research; H.S., S.S., Y.H., and F.N. performed experiments; H.S. and T.N. analyzed data; H.S. and T.N. interpreted results of experiments; H.S. and T.N. prepared figures; H.S. and T.N. drafted manuscript; H.S. and T.N. edited and revised manuscript; H.S. and T.N. approved final version of manuscript.

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