Senescence and dysfunction of proximal tubular cells are associated with activated p53 expression by indoxyl sulfate

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Am J Physiol Cell Physiol 299: C1110–C1117, 2010. First published August 18, 2010; doi:10.1152/ajpcell.00217.2010.—Various uremic toxins such as indoxyl sulfate are associated with activated p53 expression by indoxyl sulfate. Senescent and dysfunctional proximal tubular cells are associated with activated p53 expression by indoxyl sulfate. Indoxyl sulfate promotes cellular senescence and fibrosis through unknown mechanisms. The present study investigates how indoxyl sulfate accelerates the progression of chronic renal failure (CRF). Here, we found that although cellular senescence and fibrosis are detectable in the kidneys of CRF rats, the oral adsorbent AST-120 repressed these effects. Here, we found that β-galactosidase, p53 and α-SMA expression. We previously demonstrated that although cellular senescence and fibrosis are detectable in the kidneys of CRF rats, the oral adsorbent AST-120 repressed these effects. Here, we found that β-galactosidase, p53 and α-SMA were expressed and colocalized in the renal tubules of CRF rats; whereas AST-120 decreased the expression of these genes. Taken together, these findings indicate that indoxyl sulfate induces the expression and phosphorylation of p53 through ROS production, thus inhibiting cell proliferation and promoting cellular senescence and renal fibrosis.

**MATERIALS AND METHODS**

**Reagents.** Antibodies were obtained from the following suppliers: anti-β-gal, Promega (Madison, WI), anti-α-tubulin, Calbiochem (La Jolla, CA), anti-α-SMA, Sigma Chemical (St. Louis, MO), anti-p53,
**Fig. 1.** Indoxyl sulfate (IS) suppresses serum-induced proliferation of HK-2 cells. Serum-starved HK-2 cells were incubated with and without IS (250 μM) for 48 h. IS was removed, and cells were incubated with or without 10% FBS for 72 h. Proliferation was assessed as shown in MATERIALS AND METHODS. Data are shown as means ± SE of four separate experiments. *P < 0.05 vs. untreated cells. #P < 0.05 vs. FBS-treated cells.

**Fig. 2.** IS induces activated p53 expression, which is involved in suppression of serum-induced proliferation of HK-2 cells caused by IS. A: serum-starved HK-2 cells were incubated with IS (250 μM) for indicated periods and then purified mRNA was analyzed by real-time PCR using p53 primer. Amounts of p53 mRNA were evaluated as described in MATERIALS AND METHODS, and results normalized to amounts of GAPDH are expressed as ratios (fold increase) of control value. Data are shown as means ± SE of five independent experiments. *P < 0.05 vs. untreated cells. B: serum-starved HK-2 cells were incubated with IS (250 μM) for 48 h, and then cell lysates were Western blotted using anti-p53 antibody or anti-phospho-p53 (Ser-15) antibody as described in MATERIALS AND METHODS. C: intensity of p53 bands was quantified, and results normalized to amount of α-tubulin are expressed as ratios (fold increase) of control value. Data are shown as means ± SE of six independent experiments. *P < 0.05 vs. untreated cells. D: intensity of phospho-p53 (Ser-15) bands normalized to amount of α-tubulin and expressed ratios (fold increase) of control value. Data are shown as means ± SE of four independent experiments. *P < 0.05 vs. untreated cells. E: intensity of phospho-p53 bands normalized to amount of p53 and expressed as ratios (fold increase) of control value. Data are means ± SE of four independent experiments. *P < 0.05 vs. untreated cells. F: serum-starved HK-2 cells were incubated with and without IS (250 μM) for 24 h followed by IS (250 μM) for 24 h with or without pifithrin-α (PFTα, 10 μM). After IS and PFTα (10 μM) were removed, cells were incubated with or without 10% FBS for 72 h, and then proliferation was assessed as shown in MATERIALS AND METHODS. Data are shown as means ± SE of four separate experiments. *P < 0.05 vs. FBS-treated cells. #P < 0.05 vs. IS-treated cells.
Quantitative real-time PCR. Total RNA was isolated using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). 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′-CCGCAGTCAGATCTAGCG-3′ (forward) and 5′-AATCATCCATTGCTTGGGACG-3′ (reverse); α-SMA, 5′-GTGTTGCCCCTGAAGACAT-3′ (forward) and 5′-GCTGGGACATTGAAAGTCTCA-3′ (reverse); GAPDH, 5′-ATGGGGAAGGTGAAGGTCG-3′ (forward) and 5′-GGGGTCATTGATGGCAACAATA-3′ (reverse). The expression of mRNA levels was measured as the ratio of each mRNA to GAPDH mRNA.

Immunoblotting. Immunoblotting was performed as described in the supplemental data. In brief, cell lysates were fractionated by SDS-PAGE on polyacrylamide gels, and proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). p53, phospho-p53 (Ser-15), and α-SMA were detected using specific antibodies and normalized to α-tubulin. The protein bands were visualized using the Chemi-Lumi One system (Nacalai Tesque, Kyoto, Japan).

**Design of animal experiments.** Experimental rats were produced by Kureha (2). Seven-week-old male Sprague-Dawley rats (Clea, Tokyo, Japan) were used to produce CRF by 4/5-nephrectomy. Eleven weeks after 4/5-nephrectomy, the rats were randomized into two groups: control CRF rats (n = 6) and AST-120-treated CRF rats (n = 6). AST-120 was administered to the rats at a dose of 4 g/kg with powder chow (CE-2, Clea) for 16 wk, whereas powder chow alone was administered to control CRF rats. A control CRF rat died of uremia at the fifteenth week. Normal rats (n = 6) were used to compare the data with CRF rats. Body weight, blood pressure, and serum and urine parameters of these rats were described previously (2). These experiments were approved by 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.

Immunohistochemistry and Masson’s trichrome staining. Paraffin-embedded sections of kidney tissues provided by Kureha from described rat models (2) were deparaffinized and hydrated. Immunostaining was performed according to the streptavidin-biotin complex method. For immunohistochemical localization of β-gal, p53, α-SMA, and p21 in kidney tissues, sections were incubated overnight with anti-β-gal antibody at 1:50 dilution, anti-p53 antibody at 1:50, anti-α-SMA antibody at 1:100, and anti-p21 antibody at 1:50. Antigen was retrieved by microwave heating twice in 0.01 M citrate buffer (pH 6.0) for 5 min at 600 W. For evaluation of fibrosis, kidney tissues were dissected, fixed in formalin, and processed for paraffin embedding, and thin sections were stained by Masson’s trichrome (MT). All of the sections were examined under light microscopy (DN100, E600, Nikon, Tokyo, Japan).

Statistical analysis. Results are expressed as means ± SE. To compare the values between the groups, analysis of variance and Fisher’s protected least significance difference tests were performed. Results were considered statistically significant when the P value was < 0.05.

RESULTS

Indoxyl sulfate suppresses serum-induced cell proliferation by activating p53 expression. We used an indoxyl sulfate concentration of 250 μM since our previous study revealed that this is the mean serum level of indoxyl sulfate in patients on hemodialysis (23). We initially examined whether an incubation with indoxyl sulfate for 48 h regulates HK-2 cell proliferation, because indoxyl sulfate enhances the platelet-derived growth factor (PDGF)-induced proliferation of smooth muscle cells (SMC) by promoting platelet-derived growth factor receptor (PDGFR) β expression (25). Figure 1 shows that a 48-h incubation with indoxyl sulfate suppressed serum-dependent cell proliferation, although dead cells were undetectable. We then focused on p53 expression induced by indoxyl sulfate, because p53 plays a key role in suppressing cell proliferation. Figure 2A shows that indoxyl sulfate time-dependently upregu-

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Fig. 5. IS upregulates activated p53 expression through reactive oxygen species (ROS). A: serum-starved HK-2 cells were incubated with and without N-acetylcysteine (NAC, 5 mM) for 30 min, followed by IS (250 μM) for 24 h. Purified mRNA was analyzed using real-time PCR and p53 primer. Amounts of p53 mRNA were evaluated as described in MATERIALS AND METHODS, and results normalized to amount of GAPDH are expressed as ratios (fold increase) of control value. Data are shown as means ± SE of three independent experiments. *P < 0.05 vs. untreated cells. #P < 0.05 vs. IS-treated cells. B: serum-starved HK-2 cells were incubated with and without NAC (5 mM) for 30 min followed by IS (250 μM) for 24 h. Cell lysates were Western blotted using anti-p53 antibody or anti-phospho-p53 (Ser-15) antibody as described in MATERIALS AND METHODS. C: intensity of p53 bands was quantified, and results normalized to amount of α-tubulin are expressed as ratios (fold increase) of control value. Data are shown as means ± SE of six independent experiments. *P < 0.05 vs. untreated cells. #P < 0.05 vs. IS-treated cells. D: intensity of phospho-p53 (Ser-15) bands normalized to amount of α-tubulin is expressed as ratios (fold increase) of control value. Data are shown as means ± SE of four independent experiments. *P < 0.05 vs. untreated cells. #P < 0.05 vs. IS -treated cells. E: intensity of phospho-p53 bands was normalized to amount of p53 and is expressed as ratios (fold increase) of control value. Data are shown as means ± SE of four independent experiments. *P < 0.05 vs. untreated cells. #P < 0.05 vs. IS-treated cells.
lated p53 mRNA levels. In addition, Fig. 2, B–E, shows that indoxyl sulfate also increased the expression of p53 protein and of phosphorylated p53 (Ser 15) protein, of which the phosphorylation site is important for the activation of p53. To confirm that indoxyl sulfate induces activated p53 expression, we added PFTα for 24 h to cells that had been incubated with indoxyl sulfate for 24 h and then evaluated serum-induced cell proliferation. PFTα partially abrogated the indoxyl sulfate-dependent suppression of serum-induced cell proliferation (Fig. 2F). These results demonstrate that indoxyl sulfate suppresses serum-induced cell proliferation via, at least in part, by upregulating activated p53 expression.

**Indoxyl sulfate promotes cellular senescence by activating p53 expression.** We further examined the promotion of β-gal expression and SA β-gal activity by indoxyl sulfate, because SA β-gal is a marker of cellular senescence (5) and of p53 expression activated by CsA-induced senescence in HK-2 cells (11). Figure 3 shows that indoxyl sulfate time-dependently induced β-gal expression (A) and significantly increased SA β-gal activity (B and C). On the other hand, prior incubation with PFTα significantly suppressed indoxyl sulfate-induced SA β-gal activity (Fig. 4, A and B). These results demonstrate that the induction of β-gal expression correlates with increasing SA β-gal activity and that indoxyl sulfate evokes cellular senescence by activating p53 expression.

**NAC inhibited indoxyl sulfate-induced activated p53 expression.** We examined the relationship between reactive oxygen species (ROS) production and activated p53 expression using NAC, because indoxyl sulfate induces ROS production in HK-2 cells (19). Figure 5 shows that NAC inhibited indoxyl sulfate-induced p53 mRNA expression (A) and suppressed indoxyl sulfate-induced p53 protein, phosphorylated p53 protein expression and the phosphorylation levels of p53 (B–E). These results indicate that indoxyl sulfate-induced ROS increases the levels of p53 protein, phosphorylated p53 protein, and p53 phosphorylation.

**NAC and PFTα inhibited indoxyl sulfate-induced α-smooth muscle actin expression.** We previously detected a fibrosis marker gene in CRF rats (17, 18). Here, we examined the expression of α-smooth muscle actin (α-SMA), which is a marker of fibrosis. Figure 6 shows that indoxyl sulfate time-dependently upregulated α-SMA mRNA (A), whereas NAC and PFTα inhibited the indoxyl sulfate-induced expression of α-SMA mRNA and protein (B–D). These results suggest that indoxyl sulfate promotes α-SMA expression through ROS-induced activated p53 expression.

**β-Gal, p53, and α-SMA are expressed and colocalized in renal tubules of CRF rats.** We immunostained sections of the renal cortex with anti-β-gal, anti-p53, and anti-α-SMA antibodies to confirm the findings in vitro. We previously found that AST-120 suppresses indoxyl sulfate staining intensity in renal tubules and indoxyl sulfate concentrations in the serum and urine of CRF rats (16). Therefore, serum indoxyl sulfate levels decrease in AST-120-administered CRF rats. In fact, AST-120 suppresses ROS production and the expression of fibrosis marker genes in the renal tubules of CRF rats (16, 22, 26). We also recognized increased β-gal expression in the renal tubules of CRF rats compared with normal rats and AST-120-treated CRF rats (2).
The present study examined renal cortex sections from CRF rats in which β-gal expression had previously been detected in renal tubules (2), to determine whether p53 and α-SMA colocalize with β-gal at the same sites in these rats.

Figure 7 shows more intense immunoreactivity of p53, α-SMA, and β-gal proteins in the renal tubules of CRF rats than in control and AST-120-treated CRF rats. MT staining shows that the extent of interstitial fibrosis was more prominent in CRF rats than in control and AST-120-treated CRF rats (Fig. 7). These results demonstrate that β-gal, p53, and α-SMA protein levels increase in vivo as well as in vitro, and that p53 and α-SMA are colocalized with β-gal in the renal tubules of CRF rats. Furthermore, interstitial fibrosis was induced in CRF rats, and AST-120 suppressed it.

**DISCUSSION**

The present findings suggest that indoxyl sulfate positively regulates histological and functional aggravation of CRF perhaps via the following mechanisms (Fig. 8). First, indoxyl sulfate promotes activated p53 expression through ROS. The indoxyl sulfate-induced activated p53 expression sequentially suppresses serum-induced cell proliferation, elicits cellular senescence, and facilitates fibrosis. Together with the findings indicating that AST-120 inhibits expression of β-gal, p53, and α-SMA in the renal tubules of CRF rats, the present findings support the notion that indoxyl sulfate-induced activated p53 expression is implicated in one of the molecular mechanisms connecting cellular senescence with functional CRF aggravation.

We did not determine whether typical molecules that lie downstream of p53 are expressed in response to indoxyl sulfate. We examined p21 expression induced by indoxyl sulfate (see supplemental Fig. 1 at the AJP-Cell Physiology website) because p53 controls p21 expression through direct DNA binding to the p21 promoter and transactivation (8), and the p53-p21 axis regulates cell proliferation and senescence (8).
indoxyl sulfate also contributes to the induction of p21 expression and cellular effects through ROS production induced by indoxyl sulfate, because p21 expression is regulated by p53-dependent and independent pathways (10).

We previously demonstrated that the kidneys of CRF rats are dysfunctional, fibrotic, and senescent, although which indoxyl sulfate-induced factor(s) is implicated in these effects remains unknown (17, 18). The present study demonstrates that indoxyl sulfate-induced activated p53 expression regulates not only the serum-induced proliferation and activation of SA β-gal but also α-SMA expression. Although many groups have already investigated the mechanism of suppressed cell proliferation and senescence induced by p53 in detail, the molecular mechanism of α-SMA expression induced by p53 in the kidneys of CRF rats remains unclear. The present results indicate that PFTα suppresses indoxyl sulfate-induced α-SMA expression, but we did not examine whether indoxyl sulfate-activated p53 directly binds to the α-SMA promoter region and regulate α-SMA expression in the human osteogenic sarcoma cell line Saos-2. We consider that indoxyl sulfate-induced activated p53 also binds to the promoter region of α-SMA, thus inducing α-SMA expression in HK-2 cells. Therefore, activated p53 expression provoked by indoxyl sulfate might be closely related to dysfunction, senescence, and fibrosis in the kidneys of CRF rats.

AST-120 suppresses fibrosis and dysfunction in the kidney by removing the indoxyl sulfate precursor indole from the intestine and thus decreases indoxyl sulfate accumulation in the serum and renal tubules (16). AST-120 also attenuates ROS production in the renal tubules of CRF rats (22, 26). Our immunohistochemical analyses revealed that expression of β-gal, p53, and α-SMA in CRF rats are localized in the renal tubules, whereas these proteins are barely detectable in normal rats and in AST-120-treated CRF rats (Fig. 7). In addition, p21 expression in CRF rats is localized with both β-gal and p53 (Supplemental Fig. 3). These results indicate that indoxyl sulfate-induced activated p53 also binds to the promoter region and regulates α-SMA expression in the kidneys of CRF rats, and that these effects are suppressed when AST-120 decreases the indoxyl sulfate concentrations in the serum and renal tubules. Therefore, indoxyl sulfate-induced kidney dysfunction, fibrosis, and senescence might be accelerated by ROS-induced activated p53 expression in vivo as well as in vitro.

The present study is limited to in vitro and animal experiments. Clinical study is required to prove that p53-induced cell senescence is involved in the progression of renal fibrosis in CRF patients. Furthermore, the clinical effects of AST-120 on the p53-induced cell senescence in the kidneys should also be studied.

The present study shows that indoxyl sulfate-induced activated p53 expression is a critical event in the suppression of cell proliferation and in the initiation of fibrosis and senescence in proximal tubular cells. In addition to reducing indoxyl sulfate accumulation in the serum of CRF rats using AST-120, expression levels of β-gal, p53, α-SMA, and p21 were decreased in renal tubules. Therefore, understanding the process of indoxyl sulfate-dependent activated p53 expression is essential to elucidate the molecular mechanisms of indoxyl sulfate actions not only in proximal tubular cells but also in other cells such as VSMCs and ECs.
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DISCLOSURES

F. Nishijima is employed by Kureha Corporation. The other authors declared no competing interests. No conflicts of interest, financial or otherwise, are declared by the author(s).

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