Original Article

Indoxyl sulfate induces renin release and apoptosis of kidney mesangial cells

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ABSTRACT — Background: Indoxyl sulfate is considered to play a pathological role in the progression of chronic kidney disease. The aim of this study was to investigate the deleterious effects of indoxyl sulfate on kidney mesangial cells. Materials and Methods: Rat renal mesangial cells were exposed to indoxyl sulfate at a serial concentrations. Cytotoxicity of indoxyl sulfate on renal mesangial cells was determined using MTT assay. Protein levels of cleaved caspase-3, angiotensin, angiotensin converting enzyme (ACE) and renin were detected by immunoblotting. Reverse transcriptional PCR was performed to determine the mRNA expression. Results: Level of cleaved caspase-3 was augmented while the cell viability was inhibited by indoxyl sulfate in a dose-dependent manner. The mRNA expressions of pro-renin and ACE were upregulated in mesangial cells exposed to indoxyl sulfate. Level of renin and ACE was increased in response to indoxyl sulfate exposure in time-dependent fashion. Conclusion: Indoxyl sulfate increased viability and induced cell death of renal mesangial cells, which is time-dependent. The loss of cell viability is attributed to caspase-3 activity through apoptosis pathway. RAS in renal mesangial cells is activated in response to indoxyl sulfate treatment.

Key words: Indoxyl sulfate, Apoptosis, CKD, Kidney mesangial cell, Renin-angiotension-system

INTRODUCTION

Chronic kidney disease (CKD) is characterized by a progressive deterioration of kidney function that can lead to kidney failure known as end-stage renal disease (ESRD). It has been reported to be a risk factor for cardiovascular disease and to contribute to diabetes-related cardiovascular complications (Jaradat and Molitoris, 2002). CKD-related hypertension is classified into 2 groups, volume-dependent and volume-independent, based on the response to diuresis or ultrafiltration (Sica

and Carl, 2005). The latter involves an activation of renin-angiotensin systems (RAS) (Santos *et al.*, 2012). On the other hand, high blood pressure has been reported to have a strong association with the risk of both diabetic and non-diabetic renal function decline or ESRD. Elevated systemic blood pressure is shown to cause mechanical stretch of glomerular capillaries, leading to damages and repair responses of renal microvasculature (Ofstad and Iversen, 2005). Repeated injury and repair of glomerular capillaries results in glomerulosclerosis and proteinuria (Nishio *et al.*, 2002; Ishida *et al.*, 1999). It is evident

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that RAS plays a role in pathophysiology of hypertension and organ injury by regulating arterial pressure and sodium homeostasis. Recent studies have shown that aberrant activation of intrarenal RAS contributes to the development and progression of CKD and hypertension (Kagami, 2012; Wen *et al.*, 2010). All components of RAS have been reported to be expressed in various cell types of kidney (Kobori *et al.*, 2007). Proximal renal tubular cells express RAS components in response to uremic acid exposure (Sun *et al.*, 2012). Treatment of mesangial cell with H₂S leads to an activation of RAS through reactive oxygen species (ROS) (Xue *et al.*, 2013). It is suggested that intrarenal RAS could be activated in response to ROS generation.

Indoxyl sulfate is a metabolite of dietary protein resulting from the metabolism of bacterial indole in the liver. It is normally cleared by the kidney and then excreted into urine. As indoxyl sulfate level is associated with kidney function deterioration, it has been considered as a putative uremic toxin (Niwa, 2010b). Accumulation of indoxyl sulfate is found in patients with chronic kidney disease as a result of deteriorating renal function and impaired glomerular filtration (Niwa, 2010a). In turn, excessive serum indoxyl sulfate causes aberrant proliferation and fibrotic damages (Shimizu *et al.*, 2012, 2013b). Nephrotoxicity of indoxyl sulfate has been demonstrated to involve a broad range of cellular responses. It has been reported that indoxyl sulfate induces production of ROS, which in turn activate nuclear factor- κ B (NF- κ B) and p53 in renal tubular cells (Shimizu *et al.*, 2012). Moreover, exposure to indoxyl sulfate leads to an induction of senescence in a variety of cells, including nephrovascular cells (Adelibieke *et al.*, 2012). Recent studies have reported that epithelial-to-mesenchymal transition (EMT) is suggested to be involved in the development and aggravation of CKD (Sun *et al.*, 2012). Although endothelial and tubular cells are commonly used to study CKD, the renal mesangial cell providing structural support for glomerular capillary loops plays a role in progression of CKD in response to toxic injury. The mesangial cell responds to toxic challenges in several ways, including proliferation, release of proinflammatory cytokine, and fibrotic responses (Gelasco and Raymond, 2006). However, the effects of indoxyl sulfate on native glomerular cells such as mesangial cells remain sketchy.

The present study was focused on the cellular responses of kidney mesangial cell to indoxyl sulfate. We hypothesized that indoxyl sulfate could cause cell death through induction of apoptosis. In addition, activation of the intrarenal RAS in kidney mesangial cells was studied.

MATERIALS AND METHOD

Cell culture

Rat kidney mesangial cell line (CRL-2573, ATCC) was purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Basel, Switzerland) supplemented with 2% (vol./vol.) fetal calf serum (FCS; Gibco-BRL). Cells in this experiment were used within three to six passages and were examined to ensure cell exhibiting the characteristics of kidney mesangial cells.

For investigation of protein expression, cells were seeded at a density of 5×10^5 /ml in a 6-well plate and incubated with 500 μ M indoxyl sulfate (Sigma, St. Louis, MO, USA) for designated times. For investigation of ACE activity, cells were seeded at a density of 1×10^6 /ml and exposed to 500 μ M indoxyl sulfate for indicated times.

Cell viability

Cell viability was determined using MTT assay. The MTT assay depends on the extent to which viable cells convert MTT bromide to an insoluble colored formazan product that can be determined spectrophotometrically. Cells were seeded in 96-well plate at a density of 5×10^5 /ml and incubated overnight to allow cells to adhere. Cells were exposed to a serial concentration of indoxyl sulfate (0, 250, 500 and 1,000 μ M) for the designated time period. After exposure, cells were washed with PBS twice and 200 μ l of DMEM without phenol red, containing 5 mg/ml MTT, was added to each well. Three hours later, the medium was aspirated, and the converted dye was solubilized within isopropanol (0.1 N HCl in isopropanol). The resulting absorbance from each well was measured at a wavelength of 570 nm with background subtraction at 630 nm.

Annexin V-PI staining

An Annexin V (FITC-conjugated) apoptosis kit (BioVision, Mountain View, CA, USA) was used to analyze apoptotic cells. The experimental protocol followed the manufacturer's instructions. Cells (5×10^5) were cultured in 6-well plates and treated with 500 μ M indoxyl sulfate for 4, 8, 24 and 48 hr. Cells were trypsinized and washed with ice-cold PBS, which contained 0.5 ml binding buffer, 5 μ l Annexin V-FITC, and 10 μ l propidium iodide PI. After incubation for 15 min in the dark, fluorescent micrographs of the cells were taken or cells were resuspended for the flow cytometric analysis (Beckton Dickinson, Franklin Lakes, NJ, USA). The mean values of the fluorescent intensities of FITC were determined

using an FL-1 channel (488/530 ex/em nm).

Immunoblotting

Cells were washed twice with ice-cold PBS, and incubated with lysis buffer (Cell Signaling Technology, Beverly, MA, USA). The cell lysates were centrifuged at 14,000 g for 10 min at 4°C and the resulting supernatants were retained. Protein concentrations were measured using BCA™ protein assay kit (Pierce, Rockford, IL, USA). Protein samples were subjected to 12.5% SDS-polyacrylamide gel, and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The blotted membrane was blocked with 5% w/v skimmed milk in phosphate-buffered saline for 1 hr followed by an incubation with 1/1,000 dilution of the primary antibodies for 2 hr. The resulting membrane was incubated with 1/2,000 dilution of peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) and bands were revealed using an enzyme-linked chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA).

Angiotensin converting enzyme (ACE) assay

The ability to cleave the fluorogenic substrate, 7-Mca-YVADAPK (Dnp) (R&D Systems, Minneapolis, MN, USA), by purified ACE was used to assess the respective activity of ACE in the culture. Cleavage of this substrate by either enzyme removes the 2,4-dinitrophenyl moiety that quenches the fluorescence of the 7-methoxycoumarin moiety, thus resulting in increased fluorescence.

Reverse transcriptional PCR

Total RNA was extracted through cell lysing using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The purified RNA was used as a template to generate first-strand cDNA synthesis using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, St Leon-Rot, Germany). The correct size of the PCR products was checked by agarose gel electrophoresis. The primers used were listed in Table 1.

Statistical analyses

The value of each treatment group was presented as a mean with the standard deviation of triplicates. The data were compared using the One way ANOVA with Tukey-Kramer multiple comparisons post-test. The differences were considered significant for *p* values less than 0.05.

RESULTS

Indoxyl sulfate induced cytotoxicity in mesangial cells

The cytotoxicity of indoxyl sulfate in kidney mesangial cells was determined using MTT assay. Kidney mesangial cells were exposed to indoxyl sulfate at concentrations of 0, 250, 500 and 1,000 µM for different periods of time. Increased viability was observed in the kidney mesangial cells exposed to 250 and 500 µM of indoxyl sulfate for 12 hr, whereas 1,000 µM of indoxyl sulfate had no influence on kidney mesangial cells (Fig. 1A). Cytotoxic effect of indoxyl sulfate was shown in the cells after a prolonged incubation for 24 hr with a concentration of 1,000 µM (Fig. 1B). A significant decrease of the cell viability was witnessed in prolonged cultures of kidney mesangial cells with indoxyl sulfate at concentrations of 500 and 1,000 µM. Treatment with 1,000 µM of indoxyl sulfate for 48 and 72 hr inhibited of cell viability to $71.63 \pm 1.68\%$, and to $53.13 \pm 1.01\%$, respectively (Figs. 1C and 1D). Witnessing inhibition of viability, we next investigated the mechanism underlying the cytotoxic effect of indoxyl sulfate on kidney mesangial cells. We observed that the level of cleaved caspase 3 was elevated in response to indoxyl sulfate exposure (Fig. 2A). The apoptosis rates of kidney mesangial cells exposed to indoxyl sulfate 500 µM for designated time were determined through Annexin V-PI double staining by flow cytometry. Exposure to indoxyl sulfate for various incubation periods resulted in distinct apoptosis rate at 8%, 19%, 27%, and 39% in HT29 cells, respectively.

Indoxyl sulfate induces the production of renin and ACE in kidney mesangial cells

It is evident that mesangium plays a deleterious role

Table 1. Primer sequences

Gene	Forward	Reverse
ACE	5'-CTGGAGACCACTCCCATCCTTTCT-3'	5'-GATGTGGCCATCACATTCGTCAGAT-3'
Prorenin	5'-CCACCCCAAACCTTCAAAGTCG-3'	5'-TGCCACAACCCCATCAAAGTC-3'
Angiotensinogen	5'-TGTTGCTGCTGAGAAGATTG-3'	5'-CCGAGAAGTTGTCCTGGATG-3'
GAPDH	5'-GATGGCATGGACTGTGGTCA-3'	5'-GCAATGCCTCCTGCACCACC-3'

Fig. 1. Cytotoxic effects of indoxyl sulfate on kidney mesangial cells. Cells were treated with 250, 500, 1,000 μM indoxyl sulfate for (A) 12 hr, (B) 24 hr, (C) 48 hr, and (D) 72 hr. The cell survival was measured by MTT assay. Results were obtained from 3-5 experiments, and the bars represent the ratio $\pm$ S.E. (*: $P < 0.01$).

in the pathogenesis of malignant hypertension in response to toxic stress (Efrati *et al.*, 2007). We hypothesized that indoxyl sulfate stimulates renin release and ACE production in kidney mesangial cell in CKD setting. Our data showed that indoxyl sulfate upregulated the mRNA expression of ACE in kidney mesangial cells at a concentration of 500 μM in time-dependent fashion. A significant increase in mRNA level of pro-renin was detected in the cells treated with 500 μM indoxyl sulfate for 24 hr; whereas pro-renin expressions were relatively low in the group of 1,000 μM (Fig. 3). Indoxyl sulfate had no effect on the mRNA expression of angiotensin gene. We next determined the changes in protein levels of ACE and ren-

in in response to indoxyl sulfate exposure. The results showed that indoxyl sulfate induced an increase in protein level of ACE in kidney mesangial cells in a time-dependent manner (Fig. 4). The expression of renin was activated by indoxyl sulfate and reached a peak at the 12-hr time point. We also determined the level of secreted ACE by measuring the cleavage activity of ACE. The results showed that the cleavage activity of ACE was gradually increased over time in the cells treated with 500 μM indoxyl sulfate (Fig. 5). Unexpectedly, secretion of ACE induced by 1,000 μM of indoxyl sulfate did not follow the trends as that of the group of 500 μM indoxyl sulfate.

Indoxyl sulfate activates RAS

Fig. 2. Indoxyl sulfate induces apoptosis in kidney mesangial cells. (A) Changes in the expression of caspase3 protein in response to 500 μ M indoxyl sulfate were analyzed using immunoblotting. (B) Flow cytometry was done on the cells treated with indoxyl sulfate for designated times after fluorescein staining of anti-annexin V monoclonal antibody binding (FL1-H) and propidium iodide uptake (FL3-H). Results are from a representative experiment of three replicates.

DISCUSSION

In the present study, we showed that indoxyl sulfate increased cell viability after 12 hr of exposure and exerted cytotoxic effects in prolonged cultures. The inhibitory effects of indoxyl sulfate on kidney mesangial cells were found to involve activation of caspase-3 and apoptosis. Moreover, kidney mesangial cells exhibited an increased expression of ACE and renin in response to prolonged exposure of indoxyl sulfate.

Accumulating evidence has demonstrated that indoxyl sulfate induces ROS generation in a variety of cells, including renal cells (Shimizu *et al.*, 2013a; Niwa, 2010b). It is reported that indoxyl sulfate stimulates the

Fig. 3. Indoxyl sulfate activated the gene expression of renin-angiotensin-system in rat kidney mesangial cells. Polymerase chain reaction analysis shows that kidney mesangial cells had significantly increased renin and ACE mRNA expression.

Fig. 4. Indoxyl sulfate activated the protein expression of renin-angiotensin- system in rat kidney mesangial cells. Immunoblotting shows that kidney mesangial cells treated with indoxyl sulfate at concentrations of 500 and 1,000 μ M had significantly increased renin and ACE protein expression.

production of intracellular and extracellular ROS in rat mesangial cells through NADPH oxidase (Gelasco and Raymond, 2006). It has been reported that indoxyl sulfate-induced ROS activate NF- κ B, p65, and p53 expression, leading to senescence induction in proximal tubular cells (Niwa and Shimizu, 2012; Adelibieke *et al.*, 2012; Adijiang *et al.*, 2011). Moreover, the cytotoxic effect of indoxyl sulfate has been revealed to involve apoptosis induction in rat renal tubular cells through an activation of ERK1/2 and p38 MAPK (Adelibieke *et al.*, 2013). A recent study has shown that indoxyl sulfate induces AKT dephosphorylation leading to inhibited cell growth (Lin *et al.*, 2013). Our result showed that that indoxyl sulfate increased the viability of kidney mesangial cell after 12 hr

Fig. 5. Indoxyl sulfate enhance the ACE secretion in rat kidney mesangial cells. The ACE concentrations in the culture medium were measured by ELISA method. The ACE concentration of control cells was lower than detection limit. Treatment with indoxyl sulfate (500 and 1,000 μ M) for indicated time significantly increased ACE production (*: $P < 0.05$; **: $P < 0.01$, vs. control). Ctrl: control, IS: Indoxyl sulfate.

of incubation, suggesting that ROS induced by indoxyl sulfate have proliferative effect on kidney mesangial cells in a short-term incubation. A previous study in which mesangial cell was treated with gentamicin reported a similar phenomenon (Martinez-Salgado *et al.*, 2004). Consistent with previous reports, prolonged exposure to indoxyl sulfate leads to augmented level of intracellular ROS and consequently cell death. Our data showed that indoxyl sulfate inhibited cell viability of kidney mesangial cells through the caspase-3 activation. A set of experiments are required to elucidate the caspase-3 apoptotic pathway involved.

The renin-angiotensin system is reported to contribute to the pathogenesis and progression of diabetic and non-diabetic CKD. In the classic pathway of the RAS, renin is secreted from the juxtaglomerular apparatus of the kidney and acts on the angiotensinogen to generate angiotensin I (Gilbert *et al.*, 1999; Schunkert *et al.*, 1991). Angiotensin I is converted in the lungs by ACE to angiotensin II. Angiotensin II acts on the heart and the kidneys by binding to the G protein-coupled receptors type 1 and type 2 (Siragy, 2010; Wolf and Neilson, 1996). The type 1

receptor mediates the more deleterious effects of angiotensin II – that is, vasoconstriction and cardiac and vessel hypertrophy angiotensin II stimulates tubular cells to mesenchymal myofibroblast transition, interstitial fibroblast proliferation, and ECM production. In renal glomerulus, the activation of RAS has been shown to induce apoptosis leading to loss of endothelial cells and podocytes (Stitt-Cavanagh *et al.*, 2009; Liebau *et al.*, 2006), whereas it promotes proliferation of mesangial cells. Mesangial cells play a role in maintaining the normal structure and functions of glomeruli. In physiology, renal mesangial cell exhibits the target of angiotensin II and the expression of all elements in RAS. The over-activation of RAS was associated with the functional changes of mesangial cells, including stimulating cell proliferation, TGF- β and ECM productions (Cortes *et al.*, 1999; Hori *et al.*, 1998). It has been hypothesized that there is interaction between RAS and ROS. Angiotensin II stimulates ROS production by activating AT1 receptor (Chen *et al.*, 2005). ROS is recently considered as a downstream signaling molecule of angiotensin II. In the present study, we showed that indoxyl sulfate induced augmented expression of RAS components, ACE and renin, in kidney mesangial cells. It is indicated that renin release upon indoxyl sulfate exposure may play a role in deterioration of renal function through paracrine RAS. As indoxyl sulfate activate ROS production, our finding is in agreement with previous research that activation of RAS leads to ROS generation.

In conclusion, indoxyl sulfate increased viability and induced cells death of kidney mesangial cell, which is time-dependent. The loss of cell viability is attributed to caspase-3 activity through apoptosis pathway. RAS in kidney mesangial cells is activated in response to indoxyl sulfate treatment. The RAS components expressed include ACE and renin. Further study is necessary to explore role of kidney mesangial cells in indoxyl sulfate-induced renal damage and complications.

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