

# BPB Reports

## Report

### Indoxyl Sulfate Induces an Inflammatory Response in the Proximal Tubule via Macrophages

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**Indoxyl sulfate is a uremic toxin and is difficult to remove by hemodialysis owing to its tight binding to albumin in the blood. There is therefore concern that it could cause inflammatory responses in renal tissue, leading to worsening of renal failure. Recent reports suggest that indoxyl sulfate not only directly affects renal cells but also induces an inflammatory response via macrophages infiltrating renal tissues. However, the mechanism of the indoxyl-sulfate-induced inflammatory response mediated by macrophages and the effect of macrophages on renal cells have not yet been elucidated. Here, we evaluated the effect of indoxyl sulfate on the inflammatory response of macrophages and the effect of macrophages on renal cells. Indoxyl sulfate upregulated the expression of tumor necrosis factor- $\alpha$  ( $TNF-\alpha$ ) by THP-1 macrophages. Moreover, it activated the transcriptional regulator nuclear factor-kappa B (NF- $\kappa$ B) p65, and an inhibitor of NF- $\kappa$ B suppressed indoxyl-sulfate-induced  $TNF-\alpha$  elevation. Supernatant of THP-1 macrophages treated with indoxyl sulfate increased the mRNA expression levels of inflammatory cytokines in HK-2 renal proximal tubular epithelial cells. These results suggest that indoxyl sulfate increases  $TNF-\alpha$  expression through the activation of NF- $\kappa$ B in THP-1 macrophages and that macrophages stimulated with indoxyl sulfate induce an inflammatory response in the proximal tubular epithelial cells.**

**Key words** indoxyl sulfate, inflammation, macrophage, proximal tubule

## INTRODUCTION

End-stage renal disease is a condition in which renal failure progresses,<sup>1)</sup> and their main symptoms and signs are due partly to the accumulation of various uremic substances in the body as renal function declines.<sup>2,3)</sup> Uremic substances classified as serum-protein-binding small molecules are of particular concern because they bind strongly to albumin and are extremely difficult to remove from the body by hemodialysis.<sup>4)</sup> One of the serum-protein-binding small molecules is indoxyl sulfate, which is synthesized by sulfate conjugation of indole produced in the liver after the metabolism of tryptophan in food by intestinal bacteria.<sup>5)</sup> Given that the progression of renal failure is significantly faster in patients with high blood indoxyl sulfate levels,<sup>6)</sup> accumulation of indoxyl sulfate could enhance the pathology of end-stage renal disease.

From this perspective, studies aimed at elucidating the effect of indoxyl sulfate on renal failure have shown that indoxyl sulfate has a direct effect on the renal tubules.<sup>7-9)</sup> However, recent reports suggest that indoxyl sulfate not only directly affects renal cells but also induces an inflammatory response via macrophages infiltrating the renal tissue or peripheral cells constituting the kidney. In fact, indoxyl sulfate promotes macrophage infiltration in the renal cortex of rats,<sup>10)</sup>

but conversely macrophage depletion in renal failure model rats protects against deterioration of renal function and disruption of tissue structure.<sup>11)</sup> Nevertheless, the mechanism of the indoxyl-sulfate-induced inflammatory response mediated by macrophages and the effect of macrophages on renal cells have not yet been elucidated.

Here, we attempted to evaluate the effect of indoxyl sulfate on the inflammatory response of macrophages and the effect of macrophages on renal cells.

## MATERIALS AND METHODS

**Cell Line and Cell Culture** The human monocyte cell line THP-1 was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 (Wako, Osaka, Japan) with 10% fetal bovine serum (FBS, Biological Industry, Beit Haemek, Israel), 1% Antibiotic-Antimycotic ( $\times 100$ ) (Thermo Fisher Scientific, Waltham, MA, USA), and 0.1% 2-mercaptoethanol (Thermo Fisher Scientific) and maintained at 37°C at 5% CO<sub>2</sub> and > 95% humidity. THP-1 monocytes were differentiated into macrophages by the addition of 0.5  $\mu$ M phorbol-12-myristate-13-acetate (PMA, Wako Pure Chemical Industries, Osaka, Japan) for 24 h. After that, the cells were washed with phosphate-buffered

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saline (PBS), and only those that differentiated into adherent cells were used for the experiment. The human kidney proximal tubular epithelial cell line HK-2 was purchased from the American Type Culture Collection and cultured in DMEM/Ham's F-12 (Wako) with 10% FBS (Biological Industry) and 1% Antibiotic-Antimycotic ( $\times 100$ ) (Thermo Fisher Scientific) and maintained at 37°C at 5% CO<sub>2</sub> and > 95% humidity.

**Real-Time RT-PCR** THP-1 macrophages were treated with indoxyl sulfate (Sigma-Aldrich, St. Louis, MO, USA) (0.0625, 0.25, or 1 mM) for 24 h. To evaluate the effect of indoxyl-sulfate-stimulated macrophages on proximal tubular epithelial cells, HK-2 cells ( $3.0 \times 10^4$  cells/2 mL per well) seeded on to six-well plates were treated for 24 h with the 24-h culture supernatant derived from the THP-1 macrophages treated with 0, 0.0625, 0.25, or 1 mM indoxyl sulfate. For comparison, HK-2 cells were treated directly with 0, 0.0625, 0.25, or 1 mM indoxyl sulfate for 24 h. Total RNA was extracted by using a FastGene RNA Basic Kit (Nippon Genetics, Tokyo, Japan) and reverse-transcribed to cDNA by using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). A PCR mixture was prepared containing the above cDNA as a template and primers for *tumor necrosis factor- $\alpha$*  (*TNF- $\alpha$* ) (forward, 5'-GACAAGCCTGTAGCCCATGT-3' and reverse, 5'-GACAAGCCTGTAGCCCATGT-3'), *interleukin-1 $\beta$*  (*IL-1 $\beta$* ) (forward, 5'-GGACAAGCTGAGGAAGATGC-3' and reverse, 5'-TCGTTATCCCATGTGTGCGAA-3'), *monocyte chemoattractant protein-1* (*MCP-1*) (forward, 5'-ACTGAAGCTCGCACTCTC-3' and reverse, 5'-CTTGGGTTGTGGAGTGAG-3'), and  *$\beta$ -actin* (forward, 5'-GCCCTGAGCAGCTTCCA-3' and reverse, 5'-CGGATGTCCACGTCACACTTC-3') (Eurofins Genomics, Tokyo, Japan), as well as GeneAce SYBR qPCR Mix  $\alpha$  Low ROX (Nippon Gene, Tokyo, Japan). Real-time RT-PCR was performed with a CFX384 detection system (Bio-Rad Laboratories, Hercules, CA, USA).

**Western Blotting Analysis** THP-1 macrophages were treated with 0.25 mM indoxyl sulfate with or without JSH23 (Sigma-Aldrich), an inhibitor of nuclear factor- $\kappa$ B (NF- $\kappa$ B), at 0.1, 1, or 10  $\mu$ M for 24 h. Proteins were extracted by M-PER Mammalian Protein Extraction Reagent with Protease and Phosphatase Inhibitor Cocktail (both from Thermo Fisher Scientific) and mixed with Laemmli sample buffer (Bio-Rad Laboratories) containing 5% 2-Mercaptoethanol (Thermo Fisher Scientific). Samples were boiled for 5 min and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were then electro-transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and blocked with 4% (w/v) Block Ace (KAC Co., Ltd., Kyoto, Japan) in PBS plus 0.01% (v/v) Tween 20. The membranes were incubated with primary antibody overnight at 4°C and then treated with secondary antibody for 1 h at room temperature. The following antibodies were used: anti-NF- $\kappa$ B p65 monoclonal antibody (#8242, 1:1000), phospho-NF- $\kappa$ B p65 monoclonal antibody (#3033, 1:1000), anti-rabbit IgG-horseradish-peroxidase-conjugated secondary antibody (#7074, 1:2000) (Cell Signaling Technology; Danvers, MA, USA). Protein bands were detected by using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and visualized with an ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare Japan, Tokyo, Japan). The resultant images were analyzed by using ImageJ software (version 1.51, National Institutes of Health, Bethesda, MD, USA).

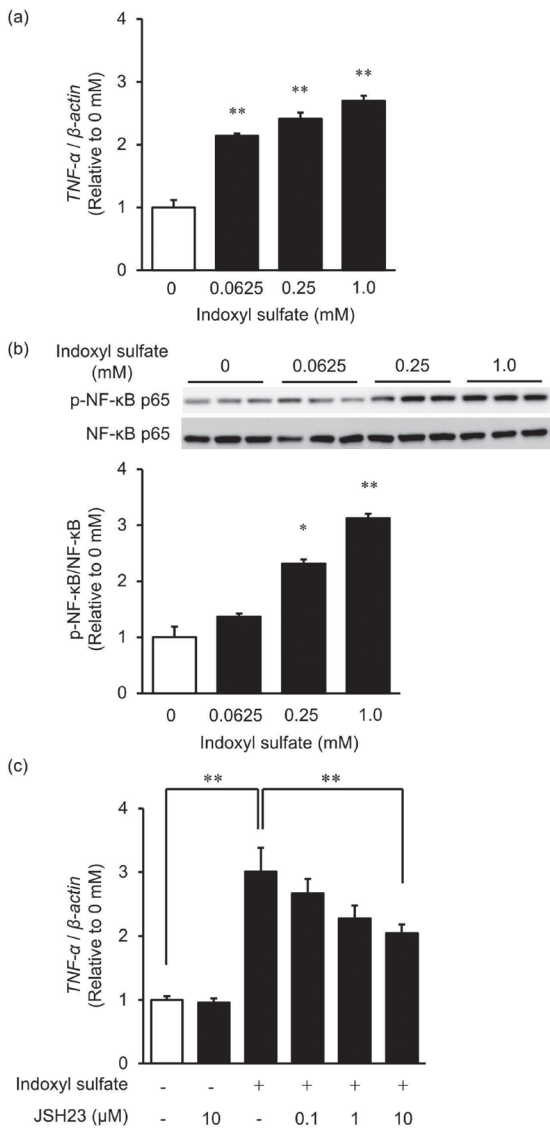
**Statistical Analysis** Statistical analyses were conducted by using Origin (OriginLab Corporation; Northampton, MA, USA). Results are expressed as means  $\pm$  S.D. Dunnett's method was used for Fig. 1a and 1b, and Tukey's method was used for Fig. 1c and 2 to compare differences. *P*-values lower than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

**Indoxyl Sulfate Induces TNF- $\alpha$  Production by Macrophages through NF- $\kappa$ B Activation** To evaluate the effects of indoxyl sulfate on the inflammatory response of macrophages, THP-1 macrophages were treated with indoxyl sulfate for 24 h and the expression of *TNF- $\alpha$*  was analyzed by real-time RT-PCR. Indoxyl sulfate treatment significantly increased the expression of *TNF- $\alpha$*  mRNA in a dose-dependent manner (Fig. 1a). Moreover, NF- $\kappa$ B, a transcription factor activated by various stimuli, activates many responses, including *TNF- $\alpha$*  signal transduction, resulting in inflammatory and immune responses.<sup>12)</sup> We therefore evaluated the involvement of NF- $\kappa$ B in the elevated *TNF- $\alpha$*  production induced by indoxyl sulfate. Western blotting analysis showed that indoxyl sulfate significantly elevated the phosphorylation of the NF- $\kappa$ B p65 subunit in a dose-dependent manner (Fig. 1b). We then co-treated THP-1 macrophages with JSH23—an inhibitor of NF- $\kappa$ B—and indoxyl sulfate for 24 h. JSH23 (10  $\mu$ M) significantly suppressed the elevation of *TNF- $\alpha$*  mRNA expression induced by indoxyl sulfate (Fig. 1c). Together, these results suggest that indoxyl sulfate increased the expression of *TNF- $\alpha$*  through the activation of NF- $\kappa$ B in THP-1 macrophages.

The aryl hydrocarbon receptor and activator protein-1 are ligand-activated transcription factors activated by several factors, including indoxyl sulfate.<sup>13–15)</sup> Kim *et al.* reported that direct interaction between NF- $\kappa$ B and the aryl hydrocarbon receptor is involved in the inflammatory response to indoxyl sulfate.<sup>16)</sup> Therefore, it should be possible to clarify the mechanism by which this occurs by evaluating the activation of transcription factors.

**Effect of Indoxyl Sulfate Exposure of Macrophages on the Tubular Inflammatory Response** Macrophages infiltrate the renal tissue—especially the renal tubular interstitium—and are involved in the exacerbation of renal failure pathology in patients with renal failure.<sup>17)</sup> This suggests that the inflammatory response of macrophages to indoxyl sulfate could affect biological responses in the proximal tubular cells and could cause the onset and progression of renal failure. We therefore assessed the effect of indoxyl sulfate on inflammatory responses in the proximal tubular epithelial cells via macrophages. HK-2 cells were treated with supernatant of THP-1 macrophages cultured with indoxyl sulfate, and changes in the mRNA expression levels of *TNF- $\alpha$*  (Fig. 2a), *IL-1 $\beta$*  (Fig. 2b), and *MCP-1* (Fig. 2c) were evaluated. In the HK-2 cells to which indoxyl sulfate was directly added, there were no significant changes in the levels of each mRNA compared with those in the cells without indoxyl sulfate. In contrast, in the HK-2 cells to which we added the supernatant of THP-1 macrophages treated with indoxyl sulfate, the expression level of each mRNA was significantly greater than that in HK-2 cells to which we added the supernatant of untreated THP-1 macrophages, and this effect had a dose-dependent trend. These results suggest that macrophages stimulated with indoxyl sulfate induced inflammatory responses in the proxi-

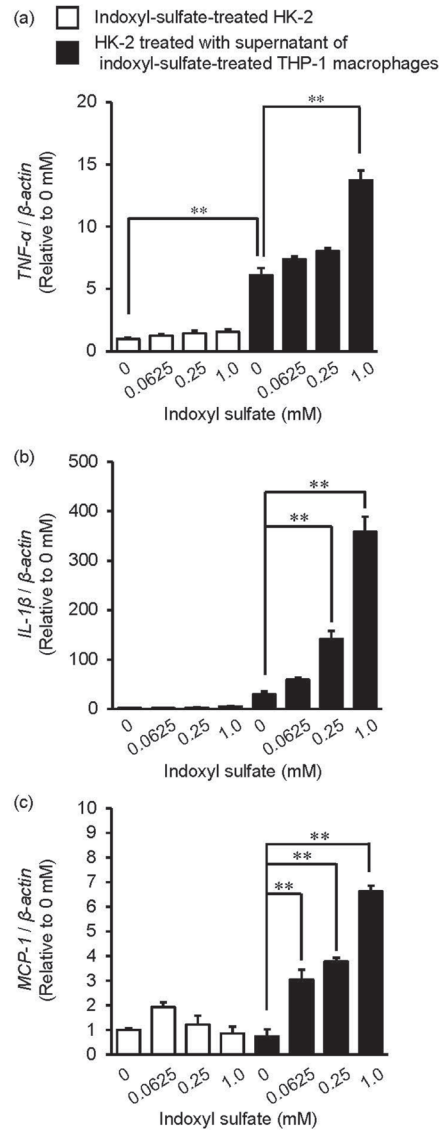


**Fig. 1.** Indoxyl Sulfate Increases TNF-α Expression Levels through NF-κB Activation in THP-1 Macrophages

THP-1 monocytes were differentiated into macrophages by treatment with phorbol-12-myristate-13-acetate (PMA) (0.5 μM) and then treated with indoxyl sulfate (0, 0.0625, 0.25, or 1 mM) for 24 h. (a) mRNA expression of *TNF-α* relative to those of *β-actin* was measured by real-time RT-PCR and (b) expression levels of the phosphorylated form of p65 (p-NF-κB p65) and the total amount of NF-κB were detected by western blotting. Band intensities were quantified from images by using ImageJ. The expression level of the phosphorylated form was corrected by the expression level of the total amount of NF-κB. (c) PMA-pretreated THP-1 macrophages were treated with indoxyl sulfate (0, 0.25 mM) with or without JSH23 (0.1, 1, or 10 μM) for 24 h. The mRNA level of *TNF-α* relative to those of *β-actin* was measured by real-time RT-PCR. All data are presented as means ± S.D.; n = 3; \*\*P < 0.01, \*P < 0.05.

mal tubular epithelial cells.

The expression of TNF-α and IL-1β was partially up-regulated in the HK-2 cells to which we added the supernatant of THP-1 macrophages treated without indoxyl sulfate. In this regard, PMA is known to induce cytokines such as TNF-α and IL-1β during the process of differentiation into THP-1 macrophages.<sup>18)</sup> Therefore, we consider that PMA-induced inflammatory responses in THP-1 macrophages might result in the enhancement of cytokine production in the HK-2 cells to which we added the supernatant of THP-1 macrophages treated without indoxyl sulfate.



**Fig. 2.** Effect of Indoxyl Sulfate Exposure of Macrophages on the Tubular Inflammatory Response

THP-1 monocytes were differentiated into macrophages by treatment with PMA (0.5 μM) and then treated with indoxyl sulfate (0, 0.0625, 0.25, or 1 mM) for 24 h. HK-2 cells were then treated for 24 h with the supernatant of these treated THP-1 macrophages or directly with indoxyl sulfate (0, 0.0625, 0.25, or 1 mM). At 24 h after treatment, mRNA levels of (a) *TNF-α*, (b) *IL-1β*, and (c) *MCP-1* relative to those of *β-actin* were measured by real time RT-PCR. All data are presented as means ± S.D.; n = 3; \*\*P < 0.01.

Our study did not identify macrophage-derived factors that induce the inflammatory response in HK-2 cells. Activator protein-1 is activated by pro-inflammatory cytokines,<sup>19)</sup> thus it is possible that the inflammatory cytokines produced by macrophages could induce an inflammatory response in HK-2 cells through the activation of transcription factors. Moreover, a comprehensive analysis of the proteins secreted by THP-1 macrophages stimulated with indoxyl sulfate would help to elucidate the inflammatory responses of renal tissue through cell-cell interaction between macrophages and proximal tubular epithelial cells.

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**Abbreviations** FBS, fetal bovine serum; IL-1 $\beta$ , interleukin-1 $\beta$ ; MCP-1, monocyte chemotactic protein-1; NF- $\kappa$ B, nuclear factor-kappa B; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; TNF- $\alpha$ , tumor necrosis factor- $\alpha$

**Conflict of interest** The authors declare no conflict of interest.

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