Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions through Up-regulation of E-selectin* §

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Despite a positive correlation between chronic kidney disease and atherosclerosis, the causative role of uremic toxins in leukocyte-endothelial interactions has not been reported. We thus examined the effects of indoxyl sulfate, a uremic toxin, on leukocyte adhesion to activated endothelial cells and the underlying mechanisms. Pretreatment of human umbilical vein endothelial cells (HUVEC) with indoxyl sulfate significantly enhanced the adhesion of human monocytes (THP-1 cell line) to TNF-α-activated HUVEC under physiological flow conditions. Treatment with indoxyl sulfate enhanced the expression level of E-selectin, but not that of ICAM-1 or VCAM-1, in HUVEC. Indoxyl sulfate treatment enhanced the activation of JNK, p38 MAPK, and NF-κB in TNF-α-activated HUVEC. Inhibitors of JNK and NF-κB attenuated indoxyl sulfate-induced E-selectin expression in HUVEC and subsequent THP-1 adhesion. Furthermore, treatment with the NAD(P)H oxidase inhibitor apocynin and the glutathione donor N-acetylcysteine inhibited indoxyl sulfate-induced enhancement of THP-1 adhesion to HUVEC. Next, we examined the in vivo effect of indoxyl sulfate in nephrectomized chronic kidney disease model mice. Indoxyl sulfate-induced leukocyte adhesion to the femoral artery was significantly reduced by anti-E-selectin antibody treatment. These findings suggest that indoxyl sulfate enhances leukocyte-endothelial interactions through up-regulation of E-selectin, presumably via the JNK- and NF-κB-dependent pathway.

Cardiovascular disease is a major cause of death in chronic kidney disease (CKD) 2 (1). Atherosclerosis is highly prevalent in patients with severe renal failure and advances more rapidly in individuals with renal dysfunction compared with the general population (2). Reduced kidney function is associated with the risk of cardiovascular events, even when the dysfunction is mild (3).

Leukocyte-endothelial interactions play an important role in the development of atherosclerosis (4). Cell adhesion molecules belonging to the immunoglobulin superfamily, such as ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), together with members of the selectin family, including E-selectin, are up-regulated to mediate monocyte/macrophage infiltration into atherosclerotic lesions (4, 5).

Indoxyl sulfate is a uremic toxin synthesized in the liver from indole, a metabolite of tryptophan produced by the intestinal flora (6). In CKD patients, the serum levels of indoxyl sulfate are increased significantly compared with those in healthy individuals (7), and a number of studies have indicated that indoxyl sulfate accelerates glomerular sclerosis, whereas its accumulation promotes renal failure (8–10).

Other studies also showed that indoxyl sulfate induces endothelial dysfunction by releasing endothelial microparticles (11) and producing reactive oxygen species (ROS) (12). However, its effect on endothelial inflammatory processes such as leukocyte recruitment to vascular endothelium has not been reported.

We report for the first time that indoxyl sulfate enhances monocyte adhesion to vascular endothelium through up-regulation of E-selectin and augmentation of oxidative stress in both in vitro and in vivo models. The underlying mechanisms seem to involve activation of JNK and NF-κB. Our findings reveal a previously unrecognized molecular link between uremic toxins and cardiovascular diseases.

**EXPERIMENTAL PROCEDURES**

Reagents—Indoxyl sulfate, N-acetylcysteine, probenecid, RPMI 1640 medium, and Dulbecco’s PBS were obtained from Sigma. The JNK phosphorylation inhibitor SP600125, the p38 MAPK phosphorylation inhibitor SB203580, the ERK1/2 inhibitor U0126, and the IκB phosphorylation inhibitor BAY11-7082 were purchased from CalBiochem. Recombinant human TNF-α was obtained from R&D Systems (Minneapolis, MN). A monoclonal antibody against E-selectin (clone 7A9) was obtained from American Type Culture Collection (Manassas, VA) (13). Antibodies against ICAM-1, VCAM-1, the NF-κB p65 subunit, and the phospho-NF-κB p65 subunit and a monoclonal blocking antibody against mouse E-selectin (clone UZ4) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ERK, anti-phospho-ERK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-JNK, and anti-phos-

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2 The abbreviations used are: CKD, chronic kidney disease; ROS, reactive oxygen species; HUVEC, human umbilical vein endothelial cell(s); IVM, intravital microscopy; OAT, organic anion transporter.
Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions

Pho-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA). Control IgM was from eBioscience (San Diego, CA). Western blotting was performed using standard protocols with ECL reagents (Amersham Biosciences).

Cell Cultures—Human umbilical vein endothelial cells (HUVEC) were purchased from Sanko Junyaku (Tokyo, Japan) and cultured in endothelial growth medium-2 (Lonza, Walkersville, MD) at 37 °C in a humidified atmosphere of 5% carbon dioxide. Plastic culture dishes were precoated with 1% (w/v) collagen, and HUVEC were used between passages 1 and 3. For use in a flow chamber apparatus, HUVEC were placed onto 22-mm fibronectin-coated glass coverslips. THP-1, a human monocytic cell line, was obtained from American Type Culture Collection, and the cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/liter l-glutamine.

Monocyte Adhesion Assay—HUVEC monolayers on coverslips were treated with various concentrations of indoxyl sulfate for 20 h and then stimulated by the addition of TNF-α for 4 h. The parallel plate flow chamber and the protocol for the adhesion assay under physiological flow conditions have been described in detail previously (14). In brief, HUVEC monolayers were positioned in a flow chamber mounted on a Nikon inverted microscope. THP-1 cells (1 × 10⁶/ml) were perfused through the chamber with a syringe pump (PHD2000, Harvard Apparatus Inc., Holliston, MA) for 10 min at a controlled flow rate to generate a shear stress of 1.0 dyne/cm². The entire period of perfusion was recorded by videotape and then transferred to a personal computer for image analysis to determine the number of adherent cells on HUVEC monolayers in 10 randomly selected ×15 microscope fields.

Luciferase Reporter Gene Assay—An NF-κB-firefly luciferase cDNA construct (pNF-κB-Luc) and a thymidine kinase-Renilla luciferase construct (pRL-TK) were obtained from Clontech. HUVEC were cultured in 24-well plates and transiently transfected using Lipofectamine LTX transfection reagents (Invitrogen). Briefly, 500 ng of the pNF-κB-Luc vector and 10 ng of the internal control pRL-TK were cotransfected into HUVEC. The culture medium was changed 4 h after transfection, and the HUVEC were incubated for another 18 h before use. The transfected HUVEC were incubated with indoxyl sulfate for 20 h and then stimulated with TNF-α (100 pg/ml) for 4 h. The firefly luciferase activity of the whole cell lysate was measured with the Dual-Luciferase reporter assay system (Promega, Madison, WI), according to the manufacturer’s protocol, using a luminometer. Renilla luciferase activity was used to normalize the activity of firefly luciferase.

Surgical Procedure—Renal failure was induced in 9-week-old male C57BL/6J (Japanese) mice (Oriental Yeast, Tokyo) or BALB/c mice (Japan Crea Laboratory, Tokyo) using two-step surgical nephrectomy as reported previously (15). Briefly, under intra-peritoneal anesthesia with sodium pentobarbital (Shering-Plough Corp., Kenilworth, NJ) at 65 mg/kg, two of the three branches of the left renal artery were ligated through a lateral incision. One week after the first operation, the right kidney was removed after ligation of the renal blood vessels and ureter under anesthesia as described above.

Four weeks after the procedure, blood and blood pressure were measured. Mice with blood urea nitrogen between 53 and 90 mg/dl and systolic blood pressure between 118 and 154 mm Hg were allocated to experimental groups. Seven weeks after the procedure, half of the mice were administered 0.065% indoxyl sulfate (200 mg/kg/day) in drinking water (referred to as Nxa+IS (nephrectomized with indoxyl sulfate treatment); n = 5), whereas the other half were given only water (Nxa; n = 5). Ten days later, leukocyte adhesion to the femoral artery was assessed by intravital microscopy (IVM) and image analyses as described previously (16). In brief, mice were injected via the left femoral vein with rhodamine 6G chloride (0.3 mg/kg in 200-300 μl of PBS; Molecular Probes) to label leukocytes in vivo. The femoral artery was found within 30 min after injection of rhodamine 6G chloride and visualized with an Olympus microscope (Model BX51WI) equipped with a water immersion objective. Adhesion of labeled leukocytes was clearly visualized on the anterior half of the vessels facing the objective. All images were recorded using a computer-assisted image analysis program (Meta-Morph). The number of adherent leukocytes (i.e. those that did not move for >3 s during the 1-min recording period) was counted along a region of interest.

The mice were killed, and whole blood samples were collected from the heart using heparinized syringes. After perfusion via the left ventricle with ice-cold PBS, the aorta was dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. For E-selectin blocking study, 1 mg/kg anti-E-selectin monoclonal antibody (n = 9) or isotype control antibody (control IgM; n = 9) was injected into the tail veins of BALB/c mice once daily for 9 consecutive days prior to IVM analysis on day 10.

Plasma Biochemistry—Plasma urea, total cholesterol, HDL cholesterol, and triglyceride measurements were performed using an automated biochemical analyzer (Spotchem™ SP-4410, Arkray Co., Kyoto, Japan). Plasma indoxyl sulfate was determined by HPLC.

Complementary DNA Preparation and Real-time Quantitative PCR—Individual mouse aortas were homogenized, and total RNA was isolated with an RNeasy mini column kit (Qiagen, Hilden, Germany). RNA purity and concentration were determined by measuring absorbance at 260 and 280 nm, respectively. cDNA was produced from 0.5 μg of RNA using a PrimeScript RT-PCR reagent kit (TAKARA BIO Inc., Kyoto) with random hexamers in 10 μl of reaction solution at 37 °C for 15 min.

Real-time quantitative RT-PCR was performed with a LightCycler (Roche Applied Science) to quantitate the mRNA expression of ICAM-1, VCAM-1, E-selectin, p47phox, p22phox, and GAPDH in mouse aortas. All primers were obtained from Qiagen. Quantitative RT-PCR was carried out using a QuantiTect™ SYBR® Green RT-PCR kit (Qiagen) with GAPDH as an internal control.

Statistical Analysis—Data are expressed as means ± S.E. One-way analysis of variance with Tukey’s post hoc test or two-tailed unpaired t test was used to estimate statistical sig-
Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions

RESULTS

Indoxyl Sulfate Enhances Monocyte Adhesion to TNF-α-Activated Vascular Endothelium—First, we examined the effects of indoxyl sulfate on leukocyte-endothelial interactions under physiological flow conditions (shear stress of 1.0 dyne/cm²). Although indoxyl sulfate did not induce significant mononuclear THP-1 cell adhesion to non-stimulated HUVEC, it significantly enhanced THP-1 cell adhesion to TNF-α-stimulated HUVEC (Fig. 1A). The effect of indoxyl sulfate became significant when HUVEC were treated with 100 pg/ml TNF-α (Fig. 1B). Considering the relatively low blood levels of TNF-α even in atherogenic conditions, our finding may indicate pathophysiologically relevant inflammatory situations in vivo. Indoxyl sulfate enhanced mononuclear THP-1 cell adhesion to TNF-α-activated HUVEC in a dose-dependent manner (Fig. 1C). Furthermore, indoxyl sulfate also enhanced the adhesion of freshly isolated human peripheral blood monocytes to HUVEC (supplemental Fig. S1).

To identify the adhesion molecules responsible for this effect of indoxyl sulfate, Western blot analysis was carried out. As shown in Fig. 1D, indoxyl sulfate enhanced TNF-α-induced E-selectin expression in HUVEC. The effect of indoxyl sulfate (2.0 mmol/liter) was observed at a TNF-α concentration as low as 25 pg/ml and became saturated at 250 pg/ml (Fig. 1D), whereas as little as 0.2 mmol/liter indoxyl sulfate significantly increased TNF-α-induced E-selectin expression (Fig. 1E). The expression of ICAM-1 and VCAM-1 was not significantly enhanced by indoxyl sulfate treatment (Fig. 1, D and E).

On the basis of these results, we incubated HUVEC with indoxyl sulfate (0.2 mmol/liter) for 20 h, followed by TNF-α (100 pg/ml) for 4 h in subsequent cell adhesion assays. Indoxyl sulfate also enhanced THP-1 cell adhesion to IL-1β-stimulated HUVEC (supplemental Fig. S2).

To confirm the importance of indoxyl sulfate-enhanced E-selectin expression, we examined adhesion assays using functional blocking antibodies against E-selectin, ICAM-1, and VCAM-1. As shown in supplemental Fig. S3, anti-E-selectin antibody, but not antibodies to ICAM-1 and VCAM-1, inhibited indoxyl sulfate-enhanced leukocyte adhesion, whereas all antibodies inhibited base-line leukocyte adhesion induced by TNF-α.

JNK Signaling Pathway Is Involved in Indoxyl Sulfate-enHanced Leukocyte-Endothelial Interactions—To further elucidate the molecular mechanisms involved in indoxyl sulfate-mediated endothelial activation, we examined the activation of MAPK family members JNK, p38 MAPK, and ERK1/2. Western blot analyses revealed that indoxyl sulfate enhanced JNK and p38 MAPK (but not ERK1/2) phosphorylation in HUVEC in the presence of TNF-α (Fig. 2A). Further experiments showed that SP600125, a chemical inhibitor of the JNK signaling pathway, abrogated the indoxyl sulfate-induced enhancement of THP-1 cell adhesion to HUVEC, whereas the p38 MAPK inhibitor SB203580 and the ERK1/2 inhibitor U0126 did not influence indoxyl sulfate-induced THP-1 cell adhesion (Fig. 2B). Moreover, SP600125 suppressed indoxyl sulfate-enhanced E-selectin expression. In contrast, neither SB203580 nor U0126 altered indoxyl sulfate-enhanced E-selectin expression (Fig. 2C).

NF-κB Signaling Pathway Is Involved in Indoxyl Sulfate-enhanced Leukocyte-Endothelial Interactions—We also examined the potential contribution of NF-κB. Indoxyl sulfate potentiated the activation of NF-κB in TNF-α-activated HUVEC, as indicated by the phosphorylation of the p65 component of NF-κB (Fig. 3A). To further confirm the direct involvement of an NF-κB-dependent pathway, HUVEC were transiently transfected with an NF-κB promoter-reporter construct. Indoxyl sulfate enhanced TNF-α-induced luciferase activity (Fig. 3B). An inhibitor of the NF-κB signaling pathway, BAY11-7082, blocked indoxyl sulfate-enhanced THP-1 cell adhesion (Fig. 3C) and E-selectin expression (Fig. 3D). Indoxyl sulfate-enhanced endothelial activation was inhibited when NF-κB was knocked down by siRNA against the NF-κB p65 subunit (supplemental Fig. S4). Although these
Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions

Indoxyl sulfate (IS) is a toxic metabolite produced in the gut of humans and has been implicated in the pathogenesis of chronic kidney disease (1). IS has been shown to induce endothelial activation and leukocytes adhesion via the TNF-α and NF-κB pathways (2). However, the mechanisms underlying these effects are not fully understood.

We investigated the role of the NAD(P)H oxidase, mitochondrial electron transport inhibitor, xanthine oxidase inhibitor, glutathione donor, and NOX2 inhibitor on IS-induced THP-1 adhesion (Fig. 5A). Both the NAD(P)H oxidase inhibitor apocynin and the glutathione donor acetylcysteine reduced IS-induced THP-1 cell adhesion to HUVECs (Fig. 4B), while the mitochondrial electron transport inhibitor rotenone failed to change the adhesion assay (data not shown).

Role of Oxidative Stress in Monocytic Cell Adhesion and E-selectin Expression—Because indoxyl sulfate has been shown to cause oxidative stress in HUVEC (12), we evaluated the role of intracellular ROS in IS-mediated endothelial activation. Staining for ROS using dihydroethidium revealed a marked increase in ROS production in IS-treated HUVEC (Fig. 4A). Both the NAD(P)H oxidase inhibitor apocynin and the glutathione donor N-acetylcycteine reduced IS-induced THP-1 cell adhesion to HUVECs (Fig. 4B) and E-selectin expression (Fig. 4C). In contrast, allopurinol (xanthine oxidase inhibitor) and rotenone (mitochondrial electron transport inhibitor) failed to change either IS-induced E-selectin expression or THP-1 cell adhesion (data not shown).

Probenecid Inhibits Indoxyl Sulfate-enhanced Monocytic Cell Adhesion and E-selectin Expression—A recent study suggested a role of cell-surface anion transporters such as organic anion transporters (OATs) in IS-mediated cell activation (17). In our experiment, we confirmed the existence of OAT1 and OAT3 in HUVEC by RT-PCR (data not shown). We then examined the effect of an inhibitor of OATs, probenecid, on IS-mediated endothelial activation. Probenecid inhibited the IS-sulfate-mediated increases in THP-1 adhesion (Fig. 5A) and E-selectin expression (Fig. 5B) but did not suppress the expression levels of ICAM-1 and VCAM-1 (data not shown).

Inhibitors reduced base-line THP-1 adhesion to activated HUVEC, they significantly inhibited IS-sulfate-enhanced adhesion (supplemental Fig. S5).

We also examined whether IS modifies TNF-α receptor expression in HUVEC. We documented that IS did not change the mRNA expression levels of TNFR1 and TNFR2 even after treatment with TNF-α (supplemental Fig. S6).

FIGURE 2. Effects of indoxyl sulfate on TNF-α-induced MAPK pathways. A, Western blot detection of phosphorylated (p) and total MAPK family members. HUVEC were incubated in the presence of the indicated concentrations of IS for 20 h and then with (+) or without (−) 0.2 mmol/liter IS for 15 min. B, adhesion assay and Western blotting for E-selectin, respectively. HUVEC were incubated with (+) or without (−) IS for 20 h and then treated with the JNK inhibitor SP600125 (10 μmol/liter), the p38 MAPK inhibitor SB203580 (5 μmol/liter), or the ERK1/2 inhibitor U0126 (10 μmol/liter) for 30 min, followed by incubation with (+) or without (−) IS (100 pg/ml) for 4 h. The cells were subjected to adhesion assays. The data from the adhesion assay are means ± S.E. (n = 10). HPF, high-power field. *, p < 0.01 versus without TNF-α; †, p < 0.01 versus with TNF-α and IS; NS, not significant. The data shown are representative of three independent experiments.

FIGURE 3. Effects of indoxyl sulfate on the TNF-α-induced NF-κB pathway. A, Western blot detection of phosphorylated (p) and total NF-κB p65 subunits. HUVEC were incubated with (+) or without (−) 0.2 mmol/liter IS for 20 h and then with (+) or without (−) TNF-α (100 pg/ml). B, the luciferase reporter assay was carried out as described under “Experimental Procedures.” The data shown are means ± S.E. of triplicate assays. *, p < 0.01 versus without TNF-α and 0 IS; †, p < 0.001 versus without TNF-α and 0 IS; ‡, p < 0.001 versus 0.2 mmol/liter IS and 0 indoxyl sulfate; †, p < 0.001 versus with TNF-α and 0 IS; ‡, p < 0.001 versus 0.2 mmol/liter IS and 0 indoxyl sulfate. C and D, adhesion assay and Western blotting for E-selectin, respectively. HUVEC were incubated with (+) or without (−) 0.2 mmol/liter IS for 20 h and then treated with the NF-κB signaling pathway inhibitor BAY11-7082 (BAY; 5 μmol/liter) for 30 min, followed by stimulation with TNF-α (100 pg/ml) for 4 h. The data from the adhesion assay are means ± S.E. (n = 10). HPF, high-power field. *, p < 0.05 versus with TNF-α and without IS; †, p < 0.01 versus with TNF-α and IS.

and TNFR2 even after treatment with TNF-α (supplemental Fig. S6).
Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions through Up-regulation of E-selectin in Vivo—Finally, we investigated whether indoxyl sulfate enhances leukocyte-endothelial interactions in vivo using a novel IVM system (16, 18). We generated nephrectomized CKD mice and allocated them to indoxyl sulfate-treated (Nx+IS) and non-treated (Nx) groups. Systolic blood pressure was elevated and plasma urea was high in both Nx and Nx+IS groups compared with normal control mice. As expected, the Nx+IS group exhibited significantly higher plasma indoxyl sulfate than the Nx group, whereas other plasma parameters, body weight, and blood pressure were not significantly different between the two groups (Table 1). IVM analysis showed that the number of leukocytes that adhered to the femoral artery was significantly increased in the Nx+IS group compared with the Nx group (Fig. 6, A and B). Simultaneous measurements of mRNA of adhesion molecules in the aorta revealed that E-selectin, but not ICAM-1 or VCAM-1, was significantly up-regulated in the Nx+IS group compared with the Nx group (Fig. 6C). The gene expression levels of the NAD(P)H oxidase subunits p47phox and p22phox were also up-regulated in the Nx+IS group (Fig. 6D).

To confirm the role of E-selectin in vivo, we utilized a functional blocking antibody against E-selectin to modulate leukocyte adhesion to the femoral artery. We injected anti-E-selectin antibody or control antibody (control IgM) into Nx+IS mice and conducted IVM analysis. Urea, lipid profile, and blood pressure did not change significantly after anti-E-selectin antibody or control antibody (control IgM) treatment (supplemental Table S1). The number of adherent leukocytes decreased in the anti-E-selectin antibody group compared with the control IgM group (Fig. 6E).

Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions

FIGURE 4. Effects of antioxidants on indoxyl sulfate-enhanced leukocyte-endothelial interactions. A, detection of ROS generation. HUVEC were treated with or without indoxyl sulfate (IS) for 2.5 h, followed by staining with dihydroethidium for 25 min at 37 °C. The upper panels show non-stimulated (non) HUVEC, and the lower panels show HUVEC treated with 0.2 mmol/liter (left panel) or 2.0 mmol/liter (right panel) indoxyl sulfate. B and C, adhesion assay and Western blotting for E-selectin, respectively, in HUVEC. Cells were treated with the glutathione donor N-acetylcysteine (NAC; 5 mmol/liter) or the NAD(P)H oxidase inhibitor apocynin (apo; 0.5 mmol/liter) for 30 min. The cells were then incubated with (+) or without (−) 0.2 mmol/liter indoxyl sulfate for 20 h, followed by stimulation with TNF-α (100 pg/ml) for 4 h. The data from the adhesion assay are means ± S.E. (n = 10). HPF, high-power field. *, p < 0.01 versus TNF and without indoxyl sulfate; †, p < 0.01 versus TNF and indoxyl sulfate. All data are representative of three independent experiments.

Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions

FIGURE 5. Effects of OAT inhibitor on indoxyl sulfate-enhanced THP-1 adhesion and E-selectin up-regulation. A and B, adhesion assay and Western blotting for E-selectin, respectively, in HUVEC. Cells were treated with the OAT inhibitor probenecid (0.2 mmol/liter) for 30 min and then incubated with (+) or without (−) 0.2 mmol/liter indoxyl sulfate (IS) for 20 h, followed by stimulation with TNF-α (100 pg/ml) for 4 h. The data from the adhesion assay are means ± S.E. (n = 10). HPF, high-power field. *, p < 0.01 versus in vivo with TNF and without indoxyl sulfate; †, p < 0.01 versus IS group with TNF and indoxyl sulfate. All data are representative of three independent experiments.

TABLE 1

Comparison of normal, Nx, and Nx+IS mice

|                   | Normal (n = 5) | Nx (n = 5) | Nx+IS (n = 5) |
|-------------------|----------------|------------|--------------|
| Body weight (g)   | 28.2 ± 0.7     | 28.0 ± 0.3 | 28.4 ± 0.4   |
| Systolic BP (mm Hg)| 119.3 ± 3.1    | 132.5 ± 3.1^a | 134.3 ± 4.2^a |
| Diastolic BP (mm Hg)| 77.9 ± 3.5     | 85.0 ± 3.2  | 83.6 ± 4.1   |
| Urea (mg/dl)      | 342 ± 2.9      | 646 ± 12.2^b | 582 ± 2.1^a  |
| Total cholesterol (mg/dl)| 62 ± 2 | 67 ± 4 | 65 ± 2 |
| HDL cholesterol (mg/dl)| 42 ± 1 | 46 ± 2 | 46 ± 2 |
| Triglyceride (mg/dl) | 57 ± 4 | 40 ± 7 | 48 ± 8 |
| Indoxyl sulfate (mg/dl) | 0.22 ± 0.03 | 0.68 ± 0.13^n | 1.57 ± 0.17^n |

*p < 0.05 versus normal mice.

^a p < 0.01 versus normal mice.

^b p < 0.01 versus Nx mice.
**Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions**

**FIGURE 6. Effects of indoxyl sulfate on leukocyte-endothelial interactions in a mouse CKD model.** A, representative images from intravital video microscopic analysis of leukocyte-adhesive interactions in femoral arteries (with the margins of vessels indicated by dotted lines) of five/six nephrectomized mice with (Nx+IS) or without (Nx) indoxyl sulfate treatment. White spots show fluorescent leukocytes labeled by intravenous injection of rhodamine 6G. B, quantitative analyses of leukocyte adhesion to the femoral arteries. Data are expressed as means ± S.E. (n = 5). C and D, quantitative real-time PCR analysis of mRNA expression of E-selectin, ICAM-1, and VCAM-1 (C) and the NAD(P)H oxidase subunits p47phox and p22phox (D) in aortas from Nx+IS (black bars) and Nx (white bars) mice compared with normal mice (gray bars). The level of mRNA expression was normalized by that of GAPDH mRNA. *, p < 0.05 versus normal mice; **, p < 0.01 versus normal mice; †, p < 0.01 versus Nx mice. E, quantitative analyses of leukocyte adhesion in femoral arteries from mice injected with anti-E-selectin antibody (anti-E-sel, black bar) or control IgM (white bar). Data are expressed as means ± S.E. (n = 9).

**DISCUSSION**

In this study, we found that pretreatment of HUVEC with indoxyl sulfate prior to incubation with TNF-α or IL-1β increased the adhesion of monocytic THP-1 cells or human peripheral blood monocytes to HUVEC in a dose-dependent manner (Fig. 1 and supplemental Fig. S1). Considering that the concentrations of indoxyl sulfate (0.2 nmol/liter) (7, 8) and TNF-α and IL-1β (19) were comparable with their blood levels observed in CKD patients, our findings indicate an important molecular background underlying the inflammatory process in CKD patients with high circulating uremic toxins. Our data may explain a recent observation by Barreto et al. (20) that serum indoxyl sulfate is associated with the incidence of vascular diseases and total mortality in CKD patients.

**Indoxyl Sulfate and Endothelial Activation**—We also found that indoxyl sulfate enhanced TNF-α- and IL-1β-activated E-selectin expression in HUVEC, which plays a dominant role in monocyte adhesion during inflammation (21, 22). Furthermore, anti-E-selectin antibody significantly inhibited leukocyte adhesion enhanced by indoxyl sulfate (supplemental Fig. S3). Thus, the present results raise the possibility that indoxyl sulfate exacerbates CKD-related vascular endothelial inflammation through up-regulation of E-selectin.

In CKD patients, monocyte/macrophage accumulation has been observed in the extracapillary areas of glomeruli (23, 24). Intense expression of E-selectin (24, 25) and activated glomerular endothelial cells (26) have been observed in the kidneys of CKD animal models. Furthermore, circulating E-selectin is a strong predictor of death and cardiovascular events in patients with end-stage renal disease (27, 28). These observations suggest an important role of E-selectin in renal deterioration.

**Indoxyl Sulfate Enhances E-selectin Expression via JNK**—Our results indicate that indoxyl sulfate mediates E-selectin overexpression through enhancement of the JNK and NF-κB signaling pathways (Figs. 2 and 3). The importance of JNK in E-selectin expression in comparison with other MAPK family members has been repeatedly emphasized (29–31). Although Kuldo et al. (31) demonstrated that inhibition of p38 MAPK diminished E-selectin expression in HUVEC activated by TNF-α for 24 h, we failed to detect any effect of a p38 MAPK inhibitor on E-selectin expression stimulated by TNF-α for 4 h.

We confirmed the expression of OAT1 and OAT3, which are molecules responsible for the incorporation of indoxyl sulfate, in HUVEC (32). Moreover, probenecid, a chemical inhibitor of OATs, suppressed indoxyl sulfate-mediated E-selectin expression in HUVEC and subsequent THP-1 cell adhesion. These results suggest that OAT1- and/or OAT3-mediated intracellular transport may be necessary for the exhibition of the indoxyl sulfate effects in HUVEC.

**Role of E-selectin in Indoxyl Sulfate-induced Inflammation in Vivo**—As demonstrated previously by others, nephrectomy per se does not induce significant vascular inflammatory reactions in mice unless they are apolipoprotein E-deficient (knock-out) (33, 34). However, indoxyl sulfate induced E-selectin expression as well as leukocyte adhesion to the femoral artery in the Nx mice. Furthermore, anti-E-selectin antibody significantly blocked leukocyte accumulation in Nx+IS mice, suggesting a dominant role of E-selectin in the leukocyte adhesion observed in the Nx mice (35). These results suggest that indoxyl sulfate induces leukocyte-endothelial interactions through up-regulation of E-selectin in vivo.

Previous studies demonstrated that indoxyl sulfate produced oxidative stress in vivo and in vitro through NAD(P)H oxidase activity (12, 36). Consistent with these observations, we documented up-regulation of p47phox and p22phox in Nx mice treated with indoxyl sulfate, suggesting a role of NAD(P)H oxidase in indoxyl sulfate-mediated oxidative stress in vivo. The therapeutic efficacy of quenching uremic toxins by oral adsorbents such as AST-120 (Kremezin, Kureha...
Indoxyl Sulfate Induces Leukocyte-Endothelial Interactions

Corp., (Tokyo) in the reduction of oxidative stress in CKD has been reported previously (36–39).

In conclusion, indoxyl sulfate enhanced leukocyte-endothelial interactions via increased selectin expression and oxidative stress in vitro and in vivo. Our data suggest a novel causative role of uremic toxins in producing vascular inflammation in patients with renal dysfunction.

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