Chronic kidney disease (CKD) is now recognized as a common condition that elevates the risk of atherosclerotic cardiovascular disease (CVD). Evidence suggests that increased oxidative stress is an emerging key mechanism of atherosclerosis in CKD. One recent study reported that indoxyl sulfate (IS), a uremic toxin derived from dietary protein, could cause vascular disorder, however, little is known about the mechanism involved. The present study examined the signaling pathway that is activated by IS to induce monocyte chemoattractant protein-1 (MCP-1), which plays an important role in the development of atherosclerosis, in cultured human umbilical vein endothelial cells (HUVEC).

Methods and Results: We show that IS enhanced reactive oxygen species (ROS) production, assessed by dihydroethidium staining, by HUVEC. IS also induced the expression of MCP-1, which was measured by enzyme-linked immunosorbent assay and real-time reverse transcription-polymerase chain reaction. These changes were suppressed by apocynin, a specific inhibitor of NADPH oxidase. Furthermore, IS induced the expression of NADPH oxidase 4 (Nox4) mRNA. IS-induced stimulation of ERK1/2 and p38 phosphorylation, detected by immunoblotting, was inhibited by apocynin. Finally, IS activated NF-κB, which was suppressed by inhibiting ERK1/2 and p38, resulting in reduced MCP-1 expression. These results suggest that IS increases NADPH oxidase-derived ROS, which in turn, activates the MAPK/NF-κB pathway and leads to induction of MCP-1 expression in HUVEC.

Conclusions: These findings raise the possibility that IS plays an important pathophysiological role in the development of CVD in individuals with CKD. (Circ J 2010; 74: 2216−2224)

Key Words: Cardiovascular disease; Chronic kidney disease; Indoxyl sulfate; Monocyte chemoattractant protein-1; Oxidative stress
uremic toxin that induces endothelial dysfunction, is associated with mortality in patients treated with hemodialysis (HD). Another study reported indoxyl sulfate (IS), which is a uremic toxin that accelerates the progression of CKD, showed a significant negative correlation with high-density lipoprotein cholesterol in 224 HD patients, using multivariate analysis. Thus, uremic toxins appear to correlate with the progression of atherosclerosis.

IS is one of the organic anions that is metabolized in the liver from indole, which is produced by intestinal bacteria as a metabolite of tryptophan. IS is normally excreted into the urine and exists at high concentrations in the serum of patients with progressive CKD. Recent studies reported that IS might be associated with endothelial dysfunction. Although it is thought that IS plays an important role in the pathophysiological conditions in patients with CKD, little is known about the mechanism involved.

To investigate the effect of IS on endothelial cells, we focused on ROS production. ROS can regulate intracellular kinase activities by reversibly inactivating phosphatases and can activate transcription factors including nuclear factor-κB (NF-κB), which in turn leads to expression of inflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1), which plays a crucial role in the recruitment of monocytes into the arterial wall. According to recent studies, ROS derived from NADPH oxidase is involved in the regulation of MCP-1 gene expression in endothelial cells. The aim of this study was to determine if IS induced ROS production by activating NADPH oxidase, leading to the induction of MCP-1 expression in human umbilical vein endothelial cells (HUVEC), and to clarify the mechanisms involved.

Methods

Reagents
Medium 199, endothelial cell growth supplement, IS, PD98059, SB203580, PTDTC, and apocynin were purchased from Sigma–Aldrich (St Louis, MO, USA). Fetal bovine serum was purchased from Biological Industries (Haemek, Israel).

Cell Culture
HUVECs were purchased from Dainippon Sumitomo Pharmacy (Osaka, Japan) and cultured in a type I collagen-coated plate (Asahi Glass, Tokyo, Japan) at 37°C and 5% CO2 in medium 199 supplemented with 10% fetal bovine serum, 10 mmol/L glutamine, 100 μg/ml heparin, 20 μg/ml endothelial growth factor, 100 μg/ml gentamicin, and 100 μg/ml amphotericin B. Cells were used for experiments between passages 4 and 7.

Determining MCP-1 Secretion
HUVECs were incubated with IS (125 μg/ml) for 1, 8 and 24 h. The supernatants were collected, and the released MCP-1 in the culture medium was quantified with sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA), according to protocol of the manufacturer.

RNA Extraction and Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) Analysis
MCP-1 mRNA expression was studied using RT and comparative PCR. Total RNA was extracted from HUVEC using ISOGEN (Nippon Gene, Toyama, Japan). The total RNA isolated by this method was undegraded and free of protein and DNA contamination. The sequences of the sense and antisense primers used for amplification were MCP-1: 5'-ACTGAAGCTCCATCTC-3', 5'-CTTGGGTTTGAGTGAG-3' and GAPDH: 5'-ACCCACCTGTTGCACGTGTA-3', 5'-ACCATCTTCAAGGAGCAGA-3'.

The PCR products were separated by electrophoresis on agarose gel in the presence of ethidium bromide and visualized under ultraviolet light.

Real-Time RT-PCR
MCP-1 and NADPH oxidase 4 (Nox4) mRNA expression was analyzed with quantitative real-time RT-PCR using an iQ5 Real-Time PCR Detection System (Bio-Rad, CA, USA) and the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad). The sequences of the sense and antisense primers used for amplification were MCP-1: 5'-TCTCAGTGCGAAGGCTCGCGA-3', 5'-GAGTGAAGTCTCAACGGTCC-3'; Nox4: 5'-CAGAAAGTTCCAACGCAGAGG-3', 5'-GTGGAAGGCGATCCAGCAGAT-3' and GAPDH: GACCCCTTGAGTGACCTCAAC-3', 5'-CTCTCCATGTTGGTGAGAAGA-3'. The fluorescent signal from SYBR Green was detected immediately after the extension step and the threshold cycle (Ct) was recorded. The Ct value from GAPDH served as an internal control for normalization.

Immunoblotting
To obtain total protein, HUVECs were homogenized in cold lysis buffer [5 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate and 5,6-μg/ml aprotinin]. The homogenates were centrifuged at 10,000 g for 30 min at 4°C, then supernatants were collected. For total nuclei, HUVECs were homogenized with 10 strokes of a Dounce homogenizer in 200 μl of ice-cold PBS supplemented with 0.5 mmol/L PMSF, 20 mmol/L NaF and 1 μg/ml of protease inhibitor cocktail. Homogenates were centrifuged at 4°C for 30 s at 12,000 g. Pellets were resuspended in 200 μl of lysis buffer [10 mmol/L HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, 20 mmol/L NaF, 20 mmol/L P-nitrophenyl phosphate, 1 mmol/L Na2VO4, 0.5 mmol/L PMSF and 1 μg/ml of protease inhibitor cocktail] and incubated on ice for 10 min. Then 10 μl of 10% IGEPAL solution was added, and the resuspended pellets were vigorously mixed for 30 s and centrifuged for 30 s at 12,000 g at 4°C. Pelleted nuclei were resuspended in 50 μl of extraction buffer [20 mmol/L HEPES, 25% glycerol, 1.5 mmol/L MgCl2, 300 mmol/L NaCl, 0.25 mmol/L EDTA, 0.5 mmol/L DTT, 20 mmol/L NaF, 20 mmol/L P-nitrophenyl phosphate, 1 mmol/L Na2VO4, 0.5 mmol/L PMSF and 1 μg/ml of protease inhibitor cocktail], incubated on ice for 20 min, and then centrifuged again for 20 min at 12,000 g at 4°C. The supernatant contained nuclear proteins. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay. Equal amounts of protein extract were separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, UK). The membrane was blocked for 1 h at room temperature with Tris-buffered saline containing 0.05% Tween 20 and 5% bovine serum albumin. After washing with TBS-T, the membranes were incubated overnight with phospho-ERK1/2 antibody (1:2,000), phospho-p38 MAPK antibody (1:2,000), NF-κBp65 antibody (1:2,000) (Cell signaling Technology Inc, Beverly, MA, USA), or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000) (GE Healthcare) and the ECL Plus sys-
tem (GE Healthcare). Images were acquired in a ChemiDoc XRS System (Bio-Rad) and analyzed with PDQuest software (Bio-Rad).

**Cellular Superoxide Detection**

Cultured HUVEC were rinsed in PBS and incubated with Dihydroethidium (DHE; 10 μmol/L) (Sigma–Aldrich) in PBS for 30 min at 37°C in a humidified chamber protected from light. Reaction with O₂⁻ oxidizes DHE to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. A 543-nm He-Ne laser was used in combination with a 560-nm long-pass filter to detect ethidium bromide.

**Statistical Analysis**

Results were expressed as mean values ± standard error of the mean (SEM). Comparisons among groups were performed by one-way ANOVA analysis. Values of P<0.05 were considered significant. Results shown are representative of at least 3 independent experiments.

**Results**

**IS Increases MCP-1 Expression in HUVEC**

It is well known that MCP-1 plays an important role in the development of atherosclerosis; however, little is known about the pathophysiological role of IS on MCP-1 in endothelial cells. We, therefore, examined how IS affected MCP-1 expression in HUVEC. At first, to determine if IS induces MCP-1 expression in vascular endothelial cells, HUVEC were incubated for 1 h in media with different concentrations of IS. Treatment of HUVEC with IS at concentrations from 25 to 125 μg/ml caused a dose-dependent increase in MCP-1 mRNA expression compared with the controls (Figures 1A, B). We performed further experiments at concentrations of 125 μg/ml, because the mean and maximal plasma concentrations of IS in uremic patients were reported to be 50 μg/ml and 240 μg/ml, respectively, and 125 μg/ml of IS significantly increased the expression of MCP-1 mRNA. IS induction of MCP-1 mRNA was time dependent, increasing within 1 h and peaking at 24 h (Figures 1C, D).

Next, we measured the release of MCP-1 protein into culture medium with an ELISA. IS stimulated the secretion of MCP-1 in HUVEC at 8 h and 24 h compared with control (Figure 1E).

**NADPH Oxidase-Derived ROS Mediates IS-Induced MCP-1 Expression in HUVEC**

As several reports have shown that ROS induces MCP-1 expression in endothelial cells, and that NADPH oxidase is a major source of superoxide in vascular cells and monocytes, we examined the effect of apocynin (600 μmol/L), a specific NADPH oxidase inhibitor, on IS-induced ROS production and MCP-1 expression. As shown in Figure 2A, HUVEC treated with IS for 2 min exhibited increased superoxide production compared with the controls, but pretreatment with apocynin for 30 min significantly reduced their ROS production, indicating that IS-induced ROS production in HUVEC was most likely derived from NADPH oxidase activity. It is reported that Nox2 and Nox4, kinds of the NADPH oxidase family, are present in vascular endothelial...

---

**Figure 1.** Indoxyl sulfate (IS) increases monocyte chemoattractant protein-1 (MCP-1) expression in human umbilical vein endothelial cells (HUVEC). HUVEC were treated with the indicated concentrations of IS for 1 h (A, B). (A) After incubating with IS, total RNA was extracted and relative MCP-1 mRNA expression was measured by reverse transcription (RT)-polymerase chain reaction (PCR). (B) MCP-1 mRNA was determined by real-time PCR. HUVEC were treated with IS (125 μg/ml) for the indicated time periods (C, D). (C) After incubating with IS, total RNA was extracted and relative MCP-1 mRNA expression was measured by RT-PCR. (D) MCP-1 mRNA was determined by real-time RT-PCR. Data are expressed as the mean±SEM. (E) HUVEC were treated with IS (125 μg/ml) for indicated time. The amount of MCP-1 protein in the culture medium was determined by enzyme-linked immunosorbent assay (ELISA). Values are means±SEM of 4 independent experiments. *P<0.05 vs control, **P<0.01 vs control, §P<0.05 vs time-matched control, §§P<0.01 vs time-matched control.
Expression of MCP-1 by Indoxyl Sulfate

by determining how components of the MAPK family, ERK1/2 and p38, were activated.

Figure 3A shows that treatment of HUVEC with IS caused a time-dependent phosphorylation of ERK1/2 with maximal levels occurring at 5 min and a return to basal levels by 60 min. As was the case for ERK1/2, p38 was phosphorylated by IS, and reached a peak at 2 min (Figure 3A). These results suggest the ERK1/2 and p38 pathways, which are associated with inflammation, are activated by IS in HUVEC.

It is known that ROS derived from NADPH oxidase can function as a signaling molecule to mediate specific cellular responses including MAP cascades. Thus, we examined the effect of apocynin on IS-induced activation of ERK1/2 and p38. As shown in Figure 3B, the IS-induced increase in ERK1/2 and p38 phosphorylation at 5 min was markedly reduced in HUVEC pretreated with apocynin for 30 min. These data indicate that NADPH oxidase-derived ROS, induced by IS, activates ERK1/2 and p38 in HUVEC.

Both ERK1/2 and p38 Are Involved in IS-Induced MCP-1 Expression in HUVEC

To explore the role of ERK1/2 and p38 in MCP-1 induction
by IS, we conducted experiments using specific inhibitors of ERK1/2 and p38. Pretreatment of HUVEC with the inhibitors of either ERK1/2 (PD98059, 30 μmol/L) or p38 (SB203580, 10 μmol/L), partially suppressed IS-induced MCP-1 mRNA expression and protein secretion. Interestingly, pretreatment of HUVEC with both PD98059 and SB20358 markedly suppressed IS-induced MCP-1 mRNA and protein (Figures 4 A–C). Taken together, these results suggest that ERK1/2 and p38 independently regulate IS-induced expression of MCP-1.

MAPK-Activated NF-κB Regulates IS-Induced MCP-1 Expression in HUVEC

It is now well appreciated that NF-κB is a redox-sensitive transcription factor that induces a number of cytokines including MCP-1.19,25,28 The major form of NF-κB is composed of a dimer of p50 and p65 subunits, which is sequestered in the cytoplasm through its tight association with specific inhibitory proteins, IκB. When a cell receives various stimulations, IκB is activated through phosphorylation. After dissociating from IκB, p65/p50 is transported into the nucleus and NF-κB target genes are transcribed.30 To evaluate the role of IS in NF-κB activation, we followed the nuclear translocation of p65 in HUVEC. Western blotting of nuclear extracts revealed that IS caused nuclear translocation of p65 within 30 min, which was detectable until at least 2 h after treatment (Figure 5A), suggesting that NF-κB is activated by IS. According to a recent study, some oxidants, including oxLDL, activate NF-κB via MAPK.22 Therefore, we examined whether inhibitors of ERK1/2 and p38 affected NF-κB activation by IS. As expected, the combination of PD98059 and SB20358 blocked IS-induced translocation of p65 into the nucleus (Figure 5B). These results suggest that IS activates NF-κB via phosphorylation of ERK1/2 and p38.

Finally, to investigate if the NF-κB pathway was involved

Figure 3. NADPH oxidase-derived reactive oxygen species (ROS) induced by indoxyl sulfate (IS) phosphorylates ERK1/2 and p38 in human umbilical vein endothelial cells (HUVEC). (A) HUVEC were treated with IS for the indicated time periods. Phosphorylation of ERK1/2 and p38 was determined by Western blotting. (B) HUVEC were pretreated with apocynin for 30 min and then incubated with IS for 5 min. Bars represent the phosphorylation level of each protein normalized to the total protein and are relative to the phosphorylation level at 0 min (determined by densitometric analysis). Values are means±SEM of 4 independent experiments. *P<0.05 vs control, **P<0.01 vs control, ***P<0.001 vs control, †P<0.05 vs IS, ††P<0.01 vs IS.
Expression of MCP-1 by Indoxyl Sulfate

in IS-induced MCP-1 expression, HUVEC were pretreated for 30 min with PDTC (10 μmol/L), an inhibitor of NF-κB. As shown in Figures 5C–E, PDTC inhibited IS-induced increases in both MCP-1 mRNA and protein, indicating that IS increases MCP-1 expression by activating NF-κB.

Discussion

The present study demonstrated that exposure of HUVEC to IS induces ROS production via NADPH oxidase, activates MAPK and NF-κB, and results in MCP-1 expression. These findings have considerable implications for atherosclerosis in the setting of CKD.

CKD is defined by the clinical practice guidelines from the National Kidney Foundation as either: (1) kidney damage for ≥3 months, as confirmed by kidney biopsy or markers of kidney damage, with or without a decrease in glomerular filtration rate (GFR), or (2) a GFR <60 mL·min⁻¹·1.73 m⁻² for ≥3 months, with or without kidney damage. The prevalence of stage 1 to 4 CKD in the USA increased from 10.0% in 1988–1994 to 13.1% in 1999–2004. As CVD is frequently associated with CKD, and individuals with CKD are more likely to die of CVD than to develop kidney failure, CKD is recognized as a worldwide health problem, and the role of individual cardiovascular risk factors in CKD are important. In individuals with CKD, CVD risk factors are classified as either ‘traditional’ or ‘non-traditional’. Traditional risk factors, such as older age, diabetes mellitus, and hypertension, are highly prevalent in CKD. But, these traditional risk factors have not fully accounted for the high risk of cardiovascular events in CKD patients, and growing evidence supports the idea that non-traditional risk factors contribute to CVD. Non-traditional risk factors include oxidative stress, inflammation, hyperhomocysteinemia, and decreased NO bioavailability. Among these, oxidative stress is involved in every step of atherosclerosis and is known to be the primary mediator that induces vascular injury.

Recently, in vitro and in vivo studies have suggested that uremic solutes, which accumulate as GFR reduces, are involved in vascular disorder. IS is a protein-bound uremic solute that is metabolized by the liver from indole, which is a tryptophan metabolite produced by the intestinal flora, and IS serum concentrations reportedly correlate with renal function. Moreover, previous studies showed that IS enhances ROS production and induced cellular toxicity in endothelial cells, vascular smooth muscle cells, glomerular mesangial cells, and renal tubular cells. Tumur and Niwa found that IS inhibited NO production and reduced cell viability by inducing ROS through induction of Nox4, a component of endothelial NADPH oxidase, in endothelial cells. Tumur et al also found that IS upregulated expression of ICAM-1 and MCP-1 by oxidative stress-
induced NF-κB activation in endothelial cells. Dou et al found that IS enhanced ROS production, increased NADPH oxidase activity, and decreased glutathione levels in endothelial cells. Although IS stimulates the production of ROS in vascular cells, little is known about its downstream signaling mechanism in human vascular endothelial cells. Therefore, the present study was designed to investigate the mechanisms underlying the modulation of MCP-1, which is a key player in the development of atherosclerosis, by IS.

At first we examined the effect of IS on MCP-1 mRNA and protein expression in HUVEC. IS significantly upregulated MCP-1 mRNA expression in a dose- and time-dependent manner, which accompanied the increased release of MCP-1 protein into the culture medium. A previous study reported that uremic patients had approximately a 50-fold increase in serum levels of IS, compared to healthy individuals, and that mean and maximal uremic concentrations were approximately 50 μg/ml and 240 μg/ml, respectively. As shown in Figures 1A and B, we observed that 25 μg/ml of IS upregulated MCP-1 expression, suggesting that IS causes endothelial damage not only in uremic patients but also in patients with more mild renal dysfunction.

Next, we analyzed the mechanisms responsible for IS-induced MCP-1 expression in HUVEC. Our results showed
Expression of MCP-1 by Indoxyl Sulfate

2223

that based on DHE staining, IS immediately increased intra-cellular ROS production in HUVEC. ROS production was blocked by apocynin, a specific NADPH oxidase inhibitor, which was accompanied by a marked reduction in MCP-1 expression.

The family of NADPH oxidases plays an important role in the production of ROS in response to receptor agonists such as growth factors or inflammatory cytokines. Nox2 and Nox4 are known to present in vascular endothelial cells. Our results showed that IS rapidly increased Nox4, but not Nox2, mRNA expression in HUVEC. These results are in agreement with previous findings that IS induced the expression of Nox4 mRNA but not Nox2 in HUVEC by Tumur and Niwa. Taken together, these observations indicate that IS-induced MCP-1 expression is mediated by ROS derived from NADPH oxidase, a major source of ROS in endothelial cells. Furthermore, our results suggest Nox4 has important role for the production of ROS by IS.

We also found that IS-induced NADPH oxidase-derived ROS activates ERK1/2 and p38 in HUVEC, because pharmacological inhibition of NADPH oxidase markedly reduced IS-induced phosphorylation. According to a previous study by Yamamoto et al., IS also activates ERK1/2 in vascular smooth muscle cells (VSMCs). Shimizu et al. also demonstrated IS induces ROS production with activation of ERK1/2 and p38 in VSMCs. Therefore, it seems that IS induces vascular damage not only in endothelial cells but also in VSMCs by activating MAPK. To further investigate if phosphorylation of ERK1/2 and p38 by ROS regulates IS-induced MCP-1 expression, we performed experiments with ERK1/2 and p38 inhibitors. We found that either PD98059 or SB203580 partially reduced IS-induced MCP-1 expression, but, more interestingly, combined treatment, with both markedly suppressed MCP-1 expression. These data suggest that the ROS-activated ERK1/2 and p38 pathways mediate IS-induced MCP-1 upregulation, synergistically.

To further clarify the downstream mechanisms by which phosphorylation of ERK1/2 and p38 regulated IS-induced MCP-1 expression, we performed experiments focused on NF-κB signaling. We demonstrated that IS induced nuclear translocation of p65, a subunit of NF-κB, within 30 min in HUVEC. To our knowledge, few reports have documented the association between IS and the MAPK/NF-κB pathway. To elucidate the mechanisms by which IS activated NF-κB, cells were pretreated with PD98059 and SB20358. We observed that IS-induced activation of NF-κB was blocked by a combination of PD98059 and SB20358, suggesting that IS activates NF-κB through phosphorylation of ERK1/2 and p38. Furthermore, inhibition of NF-κB by PDTC significantly reduced both MCP-1 mRNA and protein expression, suggesting NF-κB mediates induction of MCP-1 expression.

In summary, the current study revealed that IS exposure increased NADPH oxidase-derived ROS, which in turn, activated the MAPK (both ERK1/2 and p38)/NF-κB pathway in HUVEC, leading to induction of MCP-1, a key player in the initiation of atherosclerosis. These data clearly demonstrate that IS has significant inflammatory and pro-atherosclerotic properties in HUVEC. These findings also raise the possibility that IS plays an important pathophysiological role in the development of atherosclerotic CVD in individuals with CKD.

References


