Article

Indoxyl Sulfate–Induced Endothelial Dysfunction in Patients with Chronic Kidney Disease via an Induction of Oxidative Stress

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Summary

Background and objectives Recent data suggest indoxyl sulfate (IS), one of the uremic toxins that accelerate the progression of chronic kidney disease (CKD), may also be responsible for vascular disease via an induction of oxidative stress. The role of IS in endothelial dysfunction in CKD and potential mechanisms of IS-induced endothelial dysfunction were investigated.

Design, setting, participants, & measurements A prospective observational study in 40 CKD patients was performed. Flow-mediated endothelium-dependent vasodilatation (FMD) and its reaction time before and 24 weeks after an oral adsorbent of IS were evaluated. Plasma levels of IS and markers of oxidative stress were also measured. The proliferation, senescence, and production of nitric oxide and reactive oxygen species from human umbilical vein endothelial cells (HUVEC) were evaluated and the effect of antioxidants, *N*-acetylcysteine, rotenone, and apocynin was examined to explore the mechanism of IS-induced endothelial dysfunction.

Results AST-120 treatment for 24 weeks resulted in a significant increase in FMD with a decrease in IS and oxidized/reduced glutathione ratio. The presence of diabetes and high-sensitivity C-reactive protein were the independent predictors for an improved FMD. IS induced a production of reactive oxygen species in HUVEC, and pretreatment with antioxidants ameliorated IS-induced inhibition of proliferation and nitric oxide production and inhibited a senescence of HUVEC.

Conclusions IS may play an important role in endothelial dysfunction via generation of oxidative stress with an induction of endothelial senescence. AST-120 improved endothelial dysfunction in patients with CKD associated with a decrease in IS and a restoration of antioxidant reserve.

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Introduction

Cardiovascular disease (CVD) is a major cause of death in patients with chronic kidney disease (CKD), accounting for 50% of all deaths (approximately 9% per year), which is about 30 times the risk in the general population (1). Although recent studies have demonstrated systemic inflammatory reaction and oxidative stress as major mechanisms of vascular disease in patients with CKD (2–4), the pathophysiology of CVD in CKD has not been completely understood. Endothelial dysfunction, which was initially introduced to describe defective endothelium-dependent vasodilatation, is now regarded as one of the earliest phenomena of atherosclerotic CVD, encompassing impaired antithrombogenic property, pertubated angiogenic capacity, and inappropriate regulation of vascular tone (5). There has been a growing body of evidence showing the presence of endothelial dysfunction in patients with CKD (6–8).

Indoxyl sulfate (IS) is one of the organic anions metabolized in the liver from indole, which is produced by the intestinal bacteria as a metabolite of tryptophan derived from dietary protein (9). IS, which is increased in patients with CKD, has been known to play an important role in progression of renal damage by the mechanism of induction of inflammatory reaction and an enhanced expression of profibrotic cytokines (10–12). Administration of IS also accelerated the aggravation of renal fibrosis, whereas treatment to decrease IS level resulted in a stabilization of renal function (11–15).

In addition to its profibrotic effect, IS may also be related to the development of CVD by an inhibition of endothelial cell repair with a proliferation of vascular smooth muscle cells (16,17). Nakamura *et al.* reported that an administration of AST-120, an oral adsorbent of IS, decreased carotid intima-media thickness and pulse wave velocity in patients with CKD, which suggested the causative role of IS in the development of CVD (18).

To investigate whether IS plays a role in the development of endothelial dysfunction in CKD, we performed a clinical study to assess endothelial function by ultrasonographic evaluation of flow-mediated va-

sodilatation with an administration of AST-120 for 24 weeks, and investigated the effect of IS on proliferation, aging, and the production of nitric oxide (NO) and reactive oxygen species (ROS) in cultured human endothelial cells.

Materials and Methods
Subject and Study Design

Forty patients with CKD defined as estimated GFR (eGFR) <60 ml/min per 1.73 m^2 were enrolled. Patients with histories of active infection or hospital admission in recent 6 months and on glucocorticoid or other immunosuppressive agents were excluded from the study. Four patients dropped out from the study because of lack of compliance with medication (two cases), the diagnosis of pneumonia (one case), and colon cancer (one case) during medication. AST-120 (Kremezin; Kureha, Japan) at a dose of 6 g/d was administered for 24 weeks. Blood chemistry, plasma indoxyl sulfate, markers of oxidative stress, and endothelial function were evaluated before and 24 weeks after AST-120 treatment. To compare the endothelial function of our patients with CKD with healthy or hypertensive patients, we also measured endothelial function in two control groups comprising 12 healthy volunteers and 15 hypertensive patients matched for age, sex, body mass index, and BP who visited the Health Examination Center of Ewha Womans University Medical Center for routine checkup. The ethical committee of Ewha Womans University Medical Center approved the study, and written informed consent was obtained from all patients.

Blood and Urinary Chemistry

Blood samples were collected after overnight fasting. Blood urea nitrogen, creatinine, high-sensitivity C-reactive protein (hsCRP), uric acid, lipid profiles, and urinary protein were measured using a standard autoanalyzer (Hitachi 7600-110; Hitachi Technologies Co., Osaka, Japan). eGFR, expressed in ml/min per 1.73 m^2, was calculated using the abbreviated formula of Modification of Diet in Renal Disease (MDRD) (19).

Measurement of Plasma Indoxyl Sulfate

Plasma level of indoxyl sulfate was measured by HPLC (Agilent Technologies, Santa Clara, CA) using integration software (Hewlett-Packard Chemstation; Hewlett-Packard Inc., Germany) (20). Plasma samples were thawed at room temperature and mixed homogenously. The samples were then transferred to an autosampler and injected onto HPLC system. The separation was performed using SHISEIDO Capcell Park MF Ph-1 SC80 column (150 × 4.6 mm, 5 μm; Japan). The flow rate was set at 1.0 ml/min, and the wavelengths of the fluorescence detector were set at 295 nm for excitation and 390 nm for emission. The calibration curve was constructed at the range of 0.1 to 10.0 mg/dl.

Measurement of Parameters of Oxidative Stress

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were measured in erythrocytes using the Glutathione Assay Kit II (Cayman Chemical Co., Ann Arbor, MI) according to the protocol provided by the manufacturer. Briefly, whole blood was collected using an EDTA contained bottle. After centrifugation (1000g for 10 minutes), erythrocytes were homogenized in 1 ml of ice-cold HPLC-grade water and centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was deproteinized with 5% metaphosphoric acid (Sigma-Aldrich, St. Louis, MO) and 4 M triethanolamine (Sigma-Aldrich). The samples were then mixed with the assay cocktail reagents. After incubation at 25°C for 25 minutes, absorbance was measured at 405 nm. For measurement of GSSG exclusive of GSH, samples were derivatized by mixing with 2-vinylpyridine (Sigma-Aldrich). The inter- and intra-assay coefficients of variation were 3.6% and 1.6%, respectively. Total antioxidant activity (TAA) was measured in plasma using the Antioxidant Status Assay Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's protocol. The assay was defined as the ability of antioxidants in the plasma samples to prevent oxidation of 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) by metmyoglobin. The amount of ABTS$^+$ produced is monitored by reading the absorbance at 600 nm. The inter- and intra-assay coefficients of variation were 5.0% and 4.3%, respectively.

Measurement of Endothelial Function

The determination of endothelial function was performed according to the method of Celermajer *et al.* (21). Measurements were made by a single observer using an ultrasound system (SONOS 5500; Hewlett-Packard) and 7.5 MHz linear transducer. After 10 minutes of rest in a temperature-controlled room, the arm of each patient was comfortably immobilized in the extended position to allow consistent recording of the brachial artery 2 to 4 cm above the antecubital fossa. Three adjacent measurements of end-diastolic brachial artery diameter were made from single two-dimensional frames. A pneumatic tourniquet was inflated to 200 mmHg with obliteration of the radial pulse. After 5 minutes, the cuff was deflated rapidly and the change in luminal diameter of the brachial arteries in response to increased blood flow was measured. The maximum flow-mediated dilation (FMD) was calculated as the average of three consecutive measurements of maximum diameter. After this, the patients kept lying for 15 minutes and the changes in luminal diameter of the brachial arteries in response to 0.6 mg of nitroglycerine (endothelium-independent vasodilatation [EID]) was measured. A continuous record of B-mode scanning image of the brachial artery was recorded on Super-VHS videotape for subsequent blinded analysis. The lag times from the baseline to initial reaction and from baseline to peak reaction were measured as initial reaction time (IRT) and peak reaction time (PRT), respectively.

Isolation of Human Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh newborn umbilical veins according to the conventional protocol (22,23). The cells were treated with various concentrations of IS (0.125 to 12.5 mg/dl). Because organic anion transporter (OAT) is known to be a major carrier for transcellular transport of IS (13), we used an inhibitor of OAT, probenecid (1 mM/L), to confirm whether IS *per se* induced the changes in HUVEC. Tissue collection was approved by the Ethics Committee of the Ewha Womans University Medical Center.

Cell Proliferation and Senescence

Cell proliferation was determined by measuring [^{3}H]-thymidine incorporation and by direct cell counting as described elsewhere (23). Cell senescence was assessed by *in situ*–staining for SA-β-galactosidase as described (24). Briefly, cells were grown on six-well culture plates, washed with PBS three times, and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes. After another washing step with PBS, cells were incubated with β-galactosidase staining solution [150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid, 12 mM sodium phosphate, pH 6.0, containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)] for 48 hours at 37°C. The reaction was stopped by addition of PBS. Senescent cells were identified as blue-stained cells under light microscopy, and the percentage of SA-β-gal–positive cells were determined by counting cells in 10 random fields.

Measurement of Nitric Oxide Production

Monolayer cells were grown in six-well plate dishes until 90% confluence and then starved in a serum-free medium for 4 hours. The cells were treated with various concentrations of IS (0.25 to 2.5 mg/dl), and the supernatants were collected at different time intervals. NO release was determined by measurement of nitrite (NO_2^-) and nitrate (NO_3^-) levels using a commercially available Total NO/Nitrite/Nitrate Assay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (25).

Measurement of Reactive Oxygen Species Generation and the Effect of Antioxidants

ROS generation was determined using the peroxide-sensitive probe 2'7'-dichlorofluorescein diacetate (DCF-DA) dye as described previously (26). After treatment with IS, HUVECs were rinsed with $1 \times$ PBS and incubated with DCF-DA (10 μM) for 30 minutes. Fluorescence was monitored using a Nikon fluorescence microscope (excitation, 488 nm; emission, 610 nm) equipped with a FITC filter. The quantitative level of ROS was measured with excitation at 485 nm and emission at 530 nm by a fluorescence ELISA reader (Molecular Devices, Sunnyvale, CA). To understand the intracellular source of oxidative stress in IS-stimulated cells, cells were preincubated with three different antioxidants, *N*-acetyl cysteine (NAC, 10 mM), rotenone (1 μM, a specific inhibitor of mitochondrial function), or apocynin (100 μM, an inhibitor of NADPH oxidase) for 2 hours at 37°C with 5% CO_2, and then washed in PBS. Thereafter, the effect of these antioxidants on IS-induced changes in proliferation, senescence, and NO production of HUVEC was determined.

Statistical Analyses

All data are presented as mean $\pm$ SD or median (range) according to the pattern of data distribution. Differences in endothelial function in CKD, healthy, and hypertensive control were examined by one-way ANOVA and multiple comparison Scheffe test. Parameters before and 24 weeks after AST-120 were compared by *t* test. Spearman correlation was used to investigate potential association between variables of interest. Univariate and multivariate logistic regression analyses were conducted to identify the factors determining an improvement of FMD with AST-120. Statistical differences in the experimental data in each concentration of IS or time were compared using the Kruskal-Wallis test followed by multiple comparisons. Significance was defined as $P < 0.05$.

Results
Characteristics of Patients

Table 1 shows the baseline characteristics of the patients. Mean age of 36 patients was 49.3 (range 35 to 63) years with a duration of CKD of 42.1 months. Underlying renal diseases were diabetic nephropathy (19 cases), chronic glomerulonephritis (8 cases), hypertension (5 cases), or polycystic kidney (2 cases). Mean creatinine and eGFR estimated by the MDRD formula were 3.4 mg/dl and 21.4 ml/min per 1.73 m^2, respectively (Table 1).

Eighty-six percent of patients was on ACE inhibitor and/or angiotensin II receptor blocker, 36% was on statin, and 25% was on an antiplatelet agent such as aspirin and/or clopidogrel because of the presence of ischemic heart disease (seven cases) or history of cerebrovascular accident (two cases).

Endothelial Function in CKD Patients

Endothelial function, expressed as flow-mediated endothelial dilation (FMD), was significantly lower in patients with CKD compared with healthy control and BP-matched control with normal serum creatinine (hypertensive control). Endothelium-independent vasodilatation (EID) was also lower in patients with CKD (Table 2). IRT and PRT of FMD were delayed in patients with CKD compared with healthy and hypertensive controls. PRT of EID was also delayed in CKD, whereas IRT of EID in patients with CKD was comparable to control groups (Table 2).

Baseline FMD of patients was correlated with the duration of CKD ($r = -0.293$, $P = 0.042$). However, there was no significant association of FMD with serum levels of creatinine, IS, uric acid, hsCRP, lipid profiles, or markers of oxidative stress such as TAA,

Table 1. Patient characteristics	
Gender (man:woman)	23:13
Age (years)	49.3 ± 12.7 (35 to 63)
Duration of CKD (months)	42.1 ± 35.7 (24 to 160)
Body mass index (kg/m^2)	25.6 ± 4.1 (17.1 to 35.2)
Systolic blood pressure (mmHg)	133.1 ± 17.2 (100 to 170)
Diastolic blood pressure (mmHg)	85.5 ± 9.8 (60 to 100)
Creatinine (mg/dl)	3.4 ± 1.7 (2.4 to 4.7)
eGFR (ml/min per 1.73 m^2)	21.4 ± 9.2 (11 to 38)
Proteinuria (g/day)	1.52 ± 1.08
Ischemic heart disease	9 (25)
History of old CVA	2 (5.6)
Underlying renal disease	
diabetes	19 (52.8)
chronic glomerulonephritis	8 (22.2)
hypertension	5 (13.9)
polycystic kidney disease	2 (5.6)
unknown	2 (5.6)
Medication	
ACE inhibitor and/or ARB	31 (86)
statin	13 (36)
antiplatelet agent	9 (25)

Data expressed as mean $\pm$ SD (range) or number (%). ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker.

Table 2. Parameters of endothelial function			
	CKD ($n = 36$)	Healthy Control ($n = 12$)	Hypertensive Control ($n = 15$)
FMD (%)	3.2 (1.3 to 9.7)[a]	6.4 (2.4 to 12.5)	4.9 (1.2 to 13.8)
EID (%)	13.3 (2.2 to 20.5)[b]	19.3 (5.0 to 28.0)	12.5 (3.5 to 25.7)
IRT-FMD (seconds)	43.8 (38 to 65)[a]	28.0 (15 to 42)	27.5 (20 to 35)
IRT-EID (seconds)	92.9 (59 to 145)	85.0 (62 to 118)	90.3 (65 to 139)
PRT-FMD (seconds)	65.0 (40 to 95)[a]	35.5 (25 to 68)	36.0 (30 to 55)
PRT-EID (seconds)	245.1 (142 to 360)[a]	180.0 (150 to 205)	175.5 (135 to 210)

Data expressed as median (range).
[a]$P < 0.05$ versus healthy control and hypertensive control.
[b]$P < 0.05$ versus healthy control.

GSH, GSSG, and GSSG/GSH. Serum IS level was not correlated with duration of CKD, BP, or the markers of endothelial function and oxidative stress. In patients with CKD, those with diabetes showed a significantly lower FMD (2.9% versus 4.2%, DM versus non-DM, $P = 0.031$) with a prolonged PRT-FMD compared with those who did not have diabetes (82 seconds versus 55 seconds, DM versus non-DM, $P = 0.034$).

Effect of IS-Lowering Therapy on Endothelial Function

After 24 weeks of AST-120 (6 g/d), IS was significantly decreased (0.59 mg/dl versus 0.31 mg/dl, $P = 0.041$). However, there were no significant changes in blood urea nitrogen, creatinine, eGFR, or lipid profiles (Table 3). Interestingly, GSSG/GSH ratio, a marker of oxidative stress, was decreased with AST-

120, suggesting that antioxidant reserve might be restored with AST-120 treatment.

AST-120 administration resulted in a significant increase in FMD (3.24% versus 4.01%, $P = 0.009$; Figure 1); however, there was no significant change in EID. IRT and PRT of FMD were also improved with AST-120. Interestingly, the changes in IS (ΔIS) after 24 weeks were correlated with the changes in FMD (ΔFMD) with a marginal statistical significance ($r = 0.225$, $P = 0.06$; Figure 2). There was no significant correlation between ΔIS and ΔGSH, ΔGSSG, or ΔGSSG/GSH.

Predictors of Improved Endothelial Dysfunction with AST-120

To find the predictor of improved endothelial function with AST-120, we divided the patients into

Table 3. Laboratory findings after AST-120 administration

	Before AST-120	After AST-120	P
Blood urea nitrogen (mg/dl)	42.3 ± 11.4	44.9 ± 16.9	0.224
Creatinine (mg/dl)	3.4 ± 1.7	3.6 ± 2.0	0.125
GFR (ml/min per 1.73 m²)	21.4 ± 9.2	19.1 ± 8.4	0.707
Proteinuria (g/d)	1.52 ± 1.08	1.59 ± 1.64	0.514
Hemoglobin (mg/dl)	12.6 ± 2.8	12.1 ± 3.9	0.935
Uric acid (mg/dl)	7.7 ± 2.3	7.9 ± 1.9	0.407
hsCRP (mg/dl)	0.15 ± 0.23	0.29 ± 0.14	0.116
Total cholesterol (mg/dl)	185.4 ± 35.6	181.7 ± 33.1	0.329
Triglyceride (mg/dl)	154.2 ± 93.7	141.3 ± 65.9	0.160
LDL cholesetrol (mg/dl)	160.5 ± 51.6	166.1 ± 50.5	0.620
HDL cholesterol (mg/dl)	47.2 ± 15.1	45.8 ± 14.5	0.744
TAA (%)	36.9 (21.5 to 59.4)	44.5 (20.2 to 92.1)	0.125
GSSG (μg/ml)	114 (73.1 to 160.2)	66 (42.3 to 115.2)	0.034
GSH (μg/ml)	189 (104.8 to 312.8)	233 (148.2 to 360.8)	0.048
GSSG/GSH	0.67 (0.20 to 1.24)	0.23 (0.12 to 0.73)	0.039
IS (mg/dl)	0.59 (0.16 to 1.82)	0.31 (0.13 to 1.35)	0.041

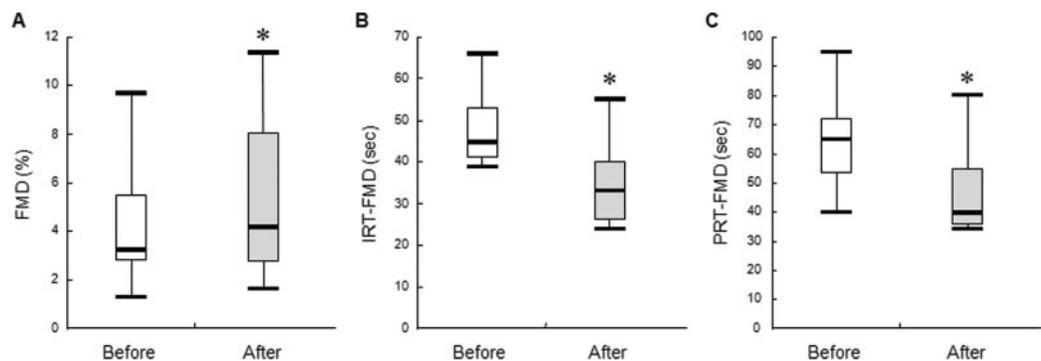Data expressed as mean ± SD or median (minimum-maximum).

Figure 1. | Endothelial function before and after IS-lowering therapy. (A) FMD, (B) IRT of FMD, and (C) PRT of FMD were improved with AST-120 for 24 weeks in patients with CKD. Horizontal lines at the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, and vertical lines above and below the boxes show the 90th and 10th percentiles, respectively. *$P < 0.05$ *versus* before AST-120.

two groups according to the changes in FMD. In patients with an increase in FMD >10% compared with pretreatment levels, baseline hsCRP was significantly lower (0.19 g/dl *versus* 0.48 g/dl, $P = 0.032$) with a lower percentage of diabetes (33% *versus* 80%, $P = 0.025$) compared with patients without a significant increase in FMD with AST-120 (Table 4). In the univariate analysis, baseline values of triglyceride, hsCRP, IRT, and PRT of FMD correlated with ΔFMD. The multivariate regression analysis including age, the presence of diabetes, eGFR, triglyceride, hsCRP, GSSG/GSH ratio, baseline FMD, and its IRT and PRT, demonstrated that both baseline hsCRP (estimate −1.922, standard error 0.750, $P = 0.016$) and the presence of diabetes (estimate −1.475, standard error 0.214, $P = 0.042$) were independent parameters determining ΔFMD with AST-120 treatment.
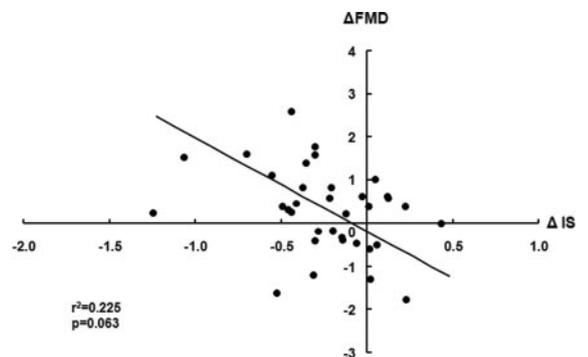

Figure 2. | Correlation between ΔIS and ΔFMD before and after AST-120.

Table 4. Comparison of baseline parameters according to the changes in FMD

	ΔFMD ≥10% (n = 21)	ΔFMD <10% (n = 15)	P
Age (years)	50.7 ± 10.4	57.4 ± 14.2	0.351
Presence of diabetes (%)	33.3	80.0	0.025
Blood urea nitrogen (mg/dl)	40.2 ± 13.8	38.5 ± 18.5	0.314
Creatinine (mg/dl)	3.2 ± 1.7	3.6 ± 2.0	0.251
GFR (ml/min per 1.73 m^2)	20.6 ± 5.6	24.1 ± 10.4	0.118
Indoxyl sulfate (mg/dl)	0.79	0.54	0.125
Uric acid (mg/dl)	6.8 ± 2.1	7.2 ± 1.7	0.345
hsCRP (mg/dl)	0.19 ± 0.24	0.48 ± 0.24	0.032
Total cholesterol (mg/dl)	171.2 ± 34.9	186.3 ± 25.8	0.310
Triglyceride (mg/dl)	144.1 ± 84.5	175.6 ± 58.3	0.248
LDL cholesterol (mg/dl)	111.5 ± 51.7	123.3 ± 25.6	0.344
HDL cholesterol (mg/dl)	45.0 ± 17.9	40.7 ± 13.1	0.231
FMD (%)	3.1	3.4	0.207
EID (%)	12.6	13.4	0.314
TAA (%)	36.5	39.5	0.230
GSSG/GSH	0.55	0.64	0.152

Data expressed as mean ± SD or median.

Role of Oxidative Stress in IS-Induced Endothelial Dysfunction in Cultured Human Vascular Endothelial Cells

To investigate the role of oxidative stress in IS-induced endothelial dysfunction, we examined the effect of IS (0.125 to 12.5 mg/dl) on proliferation, aging, and NO production in HUVECs. IS induced a dose-dependent inhibition of proliferation and an increase in cell senescence from a concentration of 0.25 mg/dl (Figure 3 and Figure 4). IS decreased NO production from 48 hours of stimulation. IS-induced changes in proliferation, senescence, or NO production were blocked by probenecid (1 mM/L), an inhibitor of organic anion transporter. IS also increased ROS generation from 10 minutes of stimulation (Figure 3D). Pretreatment of HUVEC with antioxidants, NAC (5 mM), rotenone (1 μM), or apocynin (100 μM), ameliorated IS-induced changes in cell proliferation, senescence, and the production of NO and ROS (Figure 5), suggesting that IS-induced ROS generation via an activation of NAPDH oxidase and/or mitochondrial respiration may be responsible for endothelial dysfunction by IS.

Discussion

This study demonstrated the IS might cause an endothelial dysfunction in patients with CKD (predialysis), which was supported by an improvement in FMD associated with a decrease in serum IS levels by an administration of an oral adsorbent of IS. There have been several studies suggesting the role of IS as a vascular toxin in animal models of CKD; however, this is the first showing the beneficial effect of IS-lowering therapy on early markers of endothelial dysfunction, FMD and its reaction times.

In a recent study examining the role of IS in endothelial function in animal models of CKD, a 2-week treatment of an adsorbent of IS, AST-120, resulted in an improvement of endothelium-dependent vascular response of aortic ring (27). Serum IS level significantly decreased with AST-120, whereas renal function and pathology were comparable in CKD and CKD + AST-120 rats, indicating that the beneficial effect of AST-120 on endothelial function was possibly related to a reduction of serum IS level, independent of renal function. Consistent with this finding, AST-120 treatment for 24 weeks in our study was not associated with the changes in renal function or the amount of proteinuria, but resulted in a decrease in serum IS. Moreover, a correlation between ΔIS and ΔFMD was observed, suggesting that not an improvement of renal function but a change in IS level with AST-120 was responsible for an increase in FMD.

A recent study demonstrated the IS level directly correlated with aortic calcification and vascular stiffness with an association of overall and cardiovascular mortality independent of other risk factors in patients with CKD (28,29). A clinical study in patients with CKD (predialysis) also demonstrated the decreased carotid intima-media thickness (IMT) and pulse wave velocity (PWV) with IS-lowering therapy (18). One of the most interesting findings of our study was an improvement in endothelial dysfunction with AST-120 in a relatively short treatment period of 6 months. Not only FMD but also IRT and PRT were improved with AST-120. IRT and PRT are lag times to initial and peak reaction of FMD, which were known to be sensitive markers of endothelial function assessed by Doppler ultrasonography (30). Although impaired FMD has been reported in patients with CKD (6,7,31), there is no study addressing prolonged IRT or PRT in these patients. This study is the first showing vascular reaction time and the degree of vasodilatation are
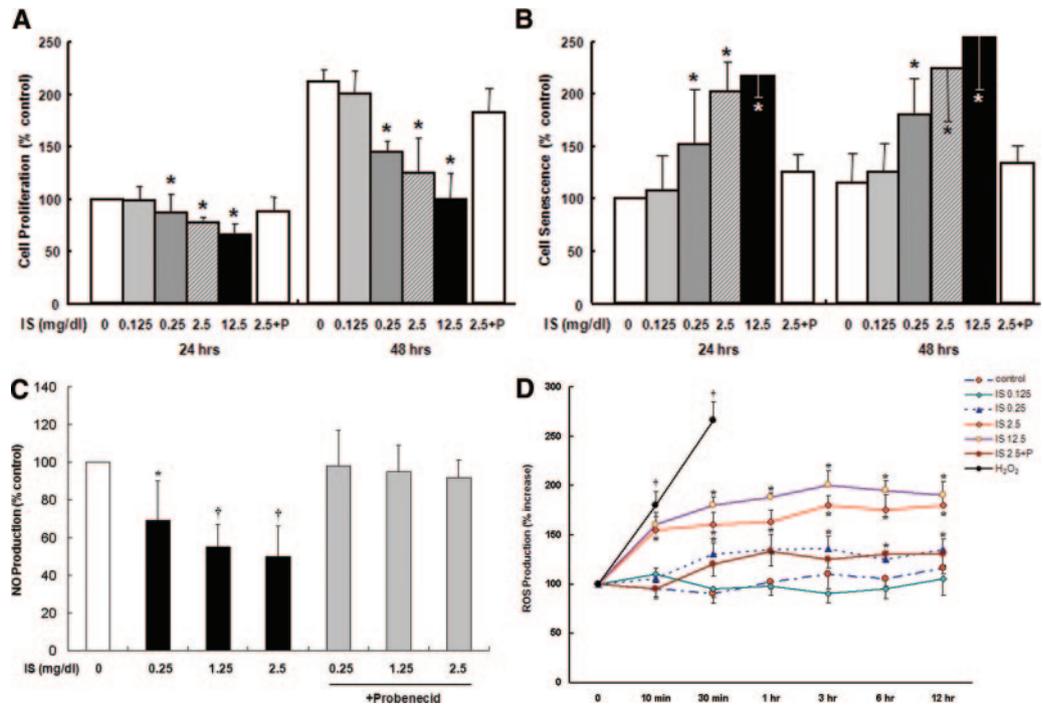
Figure 3. | Effect of IS on proliferation, senescence, and the production of NO and ROS from endothelial cells. IS induced a dose-dependent inhibition of proliferation (A), an increase in cell senescence (B), and a decrease in NO production (C) from a concentration of 0.25 mg/dl at 24 and 48 hours. IS-induced changes in HUVEC were blocked by probenecid (P). Data show the average of four separate experiments performed in duplicate. *$P < 0.05$ versus others at each time point, †$P < 0.05$ versus 0, 0.25 of IS and the conditions with probenecid. IS at the concentrations above 0.25 mg/dl also increased ROS generation from 10 minutes of stimulation (D). Hydrogen peroxide (0.1 mM) was used for positive control inducing oxidative stress in HUVEC. Data show the average of four separate experiments performed in duplicate. *$P < 0.05$ versus control (0 minutes), †$P < 0.05$ versus control and IS-containing conditions at 10 and 30 minutes.
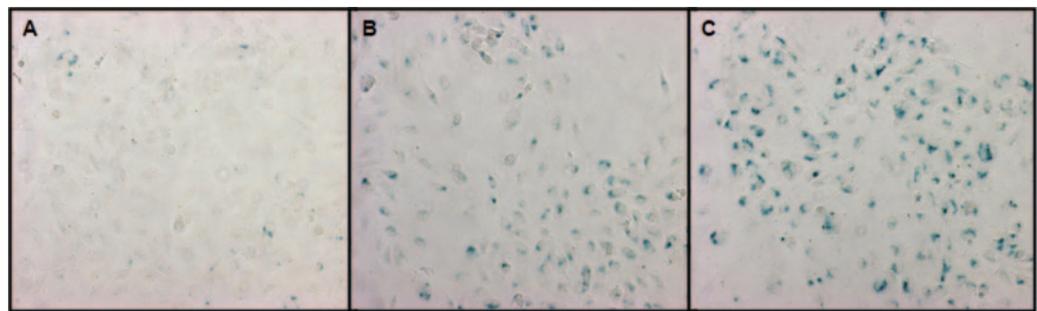

Figure 4. | IS-induced senescence of HUVEC. Representative photos of SA β-gal staining in control (A) and IS-stimulated cells (B, 2.5 mg/dl; C, 12.5 mg/dl).

impaired in CKD. In a previous study by Nakamura *et al.*, it took 12 to 24 months for the improvement of PWV and IMT with AST-120 (18). The reason why we have found the effect of AST-120 at an earlier time point than a previous study may relate to the difference in evaluated parameters of endothelial dysfunction. Although both PWV and IMT reflect the early stage of atherosclerosis, they are usually associated with anatomic and/or histopathologic changes of blood vessels including arterial wall thickening (32,33), which are preceded by the functional changes

expressed as an impaired FMD and a delayed reaction time we measured in this study.

Importantly, the presence of diabetes and inflammatory reaction seemed to hamper a favorable effect of lowering IS level on endothelial function in this study. Patients with CKD who had diabetes not only had a higher prevalence of endothelial dysfunction expressed as a lower FMD and a longer reaction time even before AST-120 treatment but also showed less changes in FMD with AST-120. Multiple regression analysis revealed that diabetes and hsCRP were two
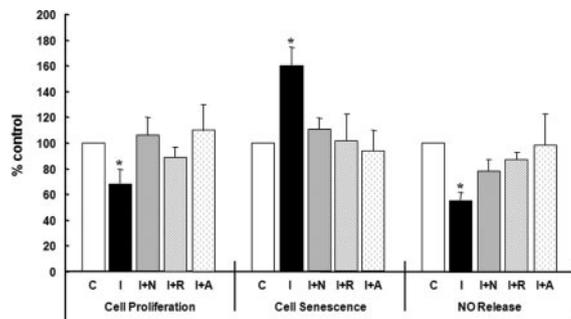
Figure 5. | Effect of antioxidants on IS-induced changes in proliferation, senescence, and NO production in HUVEC. Antioxidant, NAC (I + N), rotenone (I + R), or apocynin (I + A) ameliorated IS (0.25 mg/dl)–induced changes in cell proliferation, senescence, and NO production at 48 hours. Data show the average of six separate experiments performed in duplicate. *$P < 0.05$ *versus* others at each experiment.

independent variables determining the changes in FMD with IS-lowering therapy. It may be due to the presence of irreversible vascular damage in patients with diabetes or inflammatory reaction because both conditions are major risk factors of endothelial dysfunction and atherosclerosis (34,35).

Although the mechanism of the beneficial effect of AST-120 on markers of vascular injury is not clear, several experimental studies have shown the role of IS as a vascular toxin via the mechanisms of an induction of oxidative stress (36,37) and a decrease in NO production (38). AST-120 is an oral charcoal adsorbent that inhibits gastrointestinal absorption of aromatic compounds such as indolic and phenolic substances. This drug is not decomposed by digestive enzymes or intestinal bacteria, and is excreted into the feces within 24 hours without *in vivo* absorption or accumulation (39). Therefore, the effects of AST-120 are the consequence of an adsorption of uremic substances including IS. IS at concentrations similar to serum levels of patients with CKD has been reported to decrease NO production, which was consistent with our results performed in cultured human vascular cells. Tumur *et al.* found AST-120 treatment restored renal NO synthesis in uremic rats (38). The earliest phenomenon demonstrated in IS-stimulated human endothelial cells in our study was an induction of oxidative stress, which was found in 10 minutes of stimulation. Dou *et al.* also demonstrated that IS not only induced oxidative stress by increasing NADPH oxidase activity from endothelial cells but also significantly decreased the level of GSH, a scavenger of hydrogen peroxide and hydroxyl radical (16). Therefore, a derangement of pro- and antioxidant system in endothelial cells may be the main mechanism of IS-induced endothelial dysfunction. In this study, lowering IS by AST-120 also resulted in a decrease in markers of oxidative stress in patients with CKD. Because no single parameters of oxidative stress could fully reflect the pro- and antioxidant status in the body (40), we measured both TAA in serum and reduced/oxidized GSH in red blood cells. Re-

duced GSH is the most important antioxidant scavenging free radicals, whereas oxidized GSH (GSSG) indicates a depletion of antioxidant reserve. Increased GSSG/GSH ratio was found in patients with CKD, which was decreased with AST-120 in our study.

In an *in vitro* experiment, we also found IS *per se* inhibited the proliferation of human vascular endothelial cells with an induction of cell aging and a decrease in nitric oxide production, which were all inhibited by a pretreatment with three different antioxidants, NAC, rotenone, and apocynin. Rotenone is an agent to inhibit reactive oxygen intermediate production by inhibiting the mitochondrial electron transport system, whereas apocynin is known to block the production of superoxide completely via the inhibition of NADPH oxidase (41). NAC is a free radical scavenger and blocks the release of radicals from both the NADPH oxidase system and mitochondrial system. Therefore, IS-induced endothelial dysfunction expressed as a decrease in NO production, an inhibition of cell proliferation with senescence in our *in vitro* experiment, may be explained by IS-induced oxidative stress by an activation of both NAPDH oxidase and mitochondrial respiratory chain complex.

Importantly, IS-induced endothelial dysfunction may also play a role in the progression of renal disease in CKD. Microvascular changes in the kidney with chronic hypoxia have been regarded as a common mechanism of renal disease aggravation (42,43). Therefore, our observations suggested IS-induced oxidative stress with a decrease in NO production and endothelial senescence as one of the potential mechanisms of IS-induced progression of renal disease via an induction of renal hypoxia. A recent paper also demonstrated IS *per se* aggravated hypoxia of the kidney by dysregulating oxygen metabolism in tubular cells (44).

In conclusion, lowering serum IS level resulted in an improved endothelial dysfunction in patients with CKD (predialysis) associated with a decrease of oxidative stress markers, which could be beneficial for an improvement of both cardiovascular and renal prognosis. To the best of our knowledge, this is the first paper showing a favorable effect of IS-lowering therapy on flow-mediated vasodilatation and its reaction time in patients with CKD with a demonstration of a restoration of antioxidant reserve.

Disclosures

None.

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