Aryl Hydrocarbon Receptor Mediates Indoxyl Sulfate-Induced Cellular Senescence in Human Umbilical Vein Endothelial Cells

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Aim: Vascular senescence, which is accelerated in individuals with chronic kidney disease (CKD), contributes to the development of cardio-renal syndrome, and various uremic toxins may play important roles in the mechanisms underlying this phenomenon. We recently reported that indoxyl sulfate (IS), a uremic toxin, directly activates aryl hydrocarbon receptor (AhR) and generates oxidative stress through NADPH oxidase-4 in human umbilical vein endothelial cells (HUVECs). In the current study, we sought to examine whether IS regulates sirtuin 1 (Sirt1) and affects endothelial senescence via AhR activation.

Methods: HUVECs were incubated with 500 μmol/L of IS for the indicated time periods. In order to evaluate changes in the senescence of the HUVECs, the number of senescence-associated β-galactosidase (SAβ-gal)-positive cells was determined using an image analysis software program. The intracellular nicotinamide phosphoribosyltransferase (iNampt) activity, cellular NAD⁺/NADPH ratio and Sirt1 activity were analyzed according to a colorimetric assay to determine the mechanism of cellular senescence. Furthermore, we evaluated the involvement of AhR in the senescence-related changes induced by IS using AhR antagonists.

Results: IS decreased the iNampt activity, NAD⁺/NADPH ratio and Sirt1 activity, resulting in an increase in the percentage of SA β-gal-positive cells. On the other hand, the AhR antagonists restored the IS-induced decrease in the NAD⁺ content in association with an improvement in the iNampt activity and ameliorated the senescence-related changes. Taken together, these results indicate that IS impairs the iNampt-NAD⁺-Sirt1 system via AhR activation, which in turn promotes endothelial senescence.

Conclusions: The IS-AhR pathway induces endothelial senescence. Therefore, blocking the effects of AhR in the endothelium may provide a new therapeutic tool for treating cardio-renal syndrome.


Key words: Aryl hydrocarbon receptor, Indoxyl sulfate, Sirtuin 1, Endothelial senescence

Introduction

The correlation between chronic kidney disease (CKD) and cardiovascular disease (CVD) is termed “cardio-renal syndrome”1-3. Studies on the relationship between CVD and CKD have shown that the progression of renal dysfunction affects various factors that lead to atherosclerosis, such as those involving the renin-angiotensin system (RAS), sympathetic nervous system and oxidative stress4-6. Notably, oxidative stress has been shown to strongly contribute to the development of atherosclerosis through the effects of vascular inflammation, dysfunction and senescence7-13. The occurrence of cellular senescence in the vasculature, namely vascular senescence, has been suggested to be an important process in the development of atherosclerosis based on reports showing that senescent vascular cells are present in human atherosclerotic
stress has been shown to reduce the cellular NAD$^+$ levels of tubular and glomerular mesangial cells$^{34-38}$. Although phosphate (NADPH) oxidase in endothelial, renal cells activate nicotinamide adenine dinucleotide dative stress involves the transport of IS into the cell reported that the mechanism by which IS induces oxidized cellular senescence$^{23}$.

Sirtuin 1 (Sirt1), the closest homologue of silent information regulator2 (Sir2), has been identified to be a NAD$^+$-dependent deacetylase whose activity plays a significant role in the processes of senescence, apoptosis and cell cycle modulation by regulating the acetylation of lysine groups of many transcriptional factors and proteins, such as histones, p53 and FOXO transcriptional factors$^{17-19}$. In addition, oxidative stress has been shown to reduce the cellular NAD$^+$ content by suppressing the activation of intracellular nicotinamide phosphoribosyltransferase (iNampt), the rate-limiting enzyme for NAD$^+$ biosynthesis derived from nicotinamide (NAM), and decreasing the Sirt1 activity$^{10, 20-22}$. Therefore, Sirt1 is considered to be a key player in the promotion of oxidative stress-mediated cellular senescence$^{23}$.

Uremic toxins, accumulate in association with the progression of renal dysfunction, have been demonstrated to augment oxidative stress in CKD patients$^{9, 24-26}$. Indoxyl sulfate (IS), a protein-bound uremic toxin, is metabolized in the liver by tryptophan-derived indole, which is produced by tryptophanase in intestinal bacteria and normally excreted into the urine$^{27}$. However, a reduced renal clearance due to the progression of CKD elevates the serum level of IS, which is known to enhance oxidative stress in both humans and rats$^{27-31}$. Yu et al. demonstrated that the enhancement of oxidative stress in erythrocytes and plasma in CKD patients is correlated with the serum levels of IS$^{32}$. Studies using animal models of renal failure indicate that an elevated IS induces oxidative stress in the aorta and kidney in rats$^{13, 33}$. It has been reported that the mechanism by which IS induces oxidative stress involves the transport of IS into the cell via organic anion transporters (OAT1, OAT3), where it then activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in endothelial, renal tubular and glomerular mesangial cells$^{34-38}$. Although it is unknown how IS activates NADPH oxidase, we recently found that aryl hydrocarbon receptor (AhR), which is expressed in human umbilical vein endothelial cells (HUVECs), is involved in the upregulation of NADPH oxidase-4 induced by IS$^{39}$.

AhR is a ligand-activated nuclear receptor/transportation factor belonging to the basic helix-loophelix/per-AhR nuclear translocator (ARNT)-Sim family of proteins, the activation of which by a variety of agonists, including dioxins, is involved in drug metabolism, promoting both cancer and fetal organ development$^{40, 41}$. Recently, the activation of AhR by dioxin, a representative AhR agonist, has been reported to impair the vascular system due to increased oxidative stress. In addition, subchronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been found to mediate endothelial dysfunction due to the production of nitric oxide (NO) via the activation of AhR in the aorta in mice$^{42}$. Furthermore, the enhancement of AhR by polychlorinated biphenyls (PCBs) has been demonstrated to cause endothelial inflammation in association with an increased expression of monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion protein-1 (VCAM-1)$^{43}$. We previously found that IS activates AhR as its ligand, which in turn, induces the MCP-1 expression via the actions of mitogen-activated protein kinase (MAPK)/nuclear factor-kB (NF-kB) in HUVECs$^{39, 44}$. These reports indicate that AhR is involved in the pathogenesis of endothelial dysfunction and inflammation; however, it remains unclear whether AhR activation causes cellular senescence in endothelial cells.

The aim of the present study was therefore to examine whether the activation of AhR by IS promotes cellular senescence in HUVECs. The results demonstrated that IS-induced AhR activation impairs the iNampt-NAD$^+$-Sirt1 system due to increased oxidative stress, thus resulting in the induction of cellular senescence.

**Materials and Methods**

**Reagents**

Medium 199, endothelial cell growth supplement, IS, α-napthoflavone (ANF), CH223191 (CH), apocynin (apo) and nicotinamide (NAM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). H$_2$O$_2$ was purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Biological Industries (Haemek, Israel).

**Cell Culture**

HUVECs were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA) and cultured in a type I collagen-coated plate (Asahi Glass Tokyo, Japan) at 37°C and 5% CO$_2$ in medium 199 supplemented with 10% FBS, 10 mmol/L of glutamine, 100 μg/mL of heparin, 20 μg/mL of endothelial growth factor, 100 μg/mL of gentamicin and 100 μg/mL of amphotericin B. The cells were used for the experiments between passages 4 and 7. In order to examine
cellular senescence and senescence-related changes, the HUVECs were incubated in 60-mm dishes with IS (500 μmol/L) or H2O2 (200 μmol/L) for the indicated time periods. To determine the underlying mechanism, the cells were preincubated with ANF (10 μmol/L), CH (10 μmol/L), apo (600 μmol/L), NAM (5 mmol/L) and nothing for one hour and then stimulated with IS for 24 hours.

Senescence-Associated β-Galactosidase (SA β-gal) Staining
In order to assess the senescent changes in the phenotype of HUVECs, staining for SA β-gal, a well-established biomarker of cellular senescence, was performed. The HUVECs were incubated for 24 hours at 37°C in freshly prepared β-gal staining solution (pH 6.0) containing 1 mg/mL of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5 mmol/L of potassium ferrocyanide, 5 mmol/L of potassium ferricyanide, 150 mmol/L of NaCl, 2 mmol/L of MgCl2, 0.01% sodium deoxycholate and 0.02% Nonidet-40. The percentage of SA β-gal-positive cells was determined by counting the number of blue cells and total cells within two different fields observed under a microscope (x100) per sample. The number of cells was calculated using an image analysis software program (WinROOF; Mitani Corporation, Japan). As a positive control, H2O2 was used to induce the transition to a senescent phenotype among the HUVECs.

Sirt1 Activity Assay
HUVECs were homogenized in an ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L of NaCl, 1 mmol/L of phenylmethylsulfonyl fluoride (FMSF), 5 μg/mL of leupeptin and 1 μg/mL of aprotinin] and then centrifuged (15,000 rpm, 30 min, 4°C). The supernatant was collected, including the total proteins, after centrifugation. The lysed protein concentration in each sample was measured using a Bio-Rad detergent-compatible protein assay. A protein sample (2 or 5 μg) of each fraction was electrophoretically blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was probed with antibodies for Sirt1 and β-actin. The expression levels of specific proteins were normalized to that of β-actin in all cases. The respective fold increase over the control was then determined.

Measurement of Cellular NAD+ and NADH
The cellular NAD+/NADH Quantification Kit (Bio Vision) was used to measure the NAD+/NADH levels in the HUVECs. The kit is designed to specifically detect NAD+ and NADH based on an enzyme cycling reaction and provides a convenient method for obtaining sensitive measurements of the NAD+ and NADH levels and their ratio.

iNampt Activity Assay
The iNampt activity in the HUVECs was determined using a Nampt colorimetric assay kit (CycLex) according to the manufacturer’s instructions.

Immunoblotting
To obtain proteins, HUVECs were homogenized in ice-cold lysis buffer [1% Triton X-100, 50 mM of HEPES (pH 7.4), 100 mM of sodium pyrophosphate, 100 mM of sodium fluoride, 10 mM of EDTA, 10 mM of sodium vanadate, 1 mM of PMSF, 1 μg/mL of aprotinin and 5 μg/mL of leupeptin], and the supernatant was collected, including the total proteins, after centrifugation (15,000 rpm, 30 min, 4°C). The lysed protein concentration in each sample was measured using a Bio-Rad detergent-compatible protein assay. A protein sample (2 or 5 μg) of each fraction was electrophoretically blotted onto a polyvinylidene difluoride (PVDF) membrane. Images were acquired using a ChemiDoc XRS System (Bio-Rad) and analyzed with the PDQuest software package (Bio-Rad). The expression levels of specific proteins were normalized to that of β-actin in all cases. The respective fold increase over the control was then determined.

Statistical Analysis
The results are expressed as the mean ± standard error of the mean (SEM). Comparisons between groups were made using a one-way ANOVA analysis. P values of <0.05 were considered to be statistically significant.

Results
IS Induces Cellular Senescence by Impairing the iNampt-NAD+/-Sirt1 System in HUVECs
First, we examined whether IS accelerates cellular senescence in HUVECs. HUVECs were incubated with IS for 24 hours at a concentration of 500 μmol/L, which is often used in many studies to enhance oxidative stress in these cells13, 45, 46, after which we detected cellular senescence using SA β-gal staining. As shown in Fig. 1A, IS significantly increased the percentage of SA β-gal-positive cells, as scored by counting the numbers of blue and total cells, compared to that observed in the control cells at 24 hours,
Fig. 1. IS induces cellular senescence by impairing the iNampt-NAD⁺-Sirt1 system in HUVECs. HUVECs were treated with or without IS (500 μmol/L) for 24 hours. (A) Cellular senescence was detected using SA β-gal staining, and the percentage of SA β-gal positive cells was scored by counting the number of blue and total cells within two different fields observed under a microscope per sample. The values are presented as the mean ± standard error of the mean (SEM) of each samples (n=3). ***p<0.001 vs. Control. HUVECs were treated with IS (500 μmol/L) for the indicated time periods. (B) The Sirt1 protein expression was analyzed using immunoblotting. (C) The Sirt1 activity was detected using an HDAC colorimetric assay. (D) The NAD⁺/NADH ratio was measured using a colorimetric assay. (E) The iNampt activity was analyzed using a colorimetric assay. The values are presented as the mean ± standard error of the mean (SEM) of three independent experiments for B-E (n=4 or 10). §p<0.05, §§p<0.01 vs. 0min. IS, indoxyl sulfate; Sirt1, sirtuin 1; iNampt, intracellular nicotinamide phosphoribosyltransferase; NAD, nicotinamide adenine dinucleotide; HUVECs, human umbilical vein endothelial cells; SA β-gal, senescence-associated β-galactosidase; HDAC, histone deacetylase.
indicating that IS accelerates cellular senescence in HUVECs. Next, we examined whether Sirt1, a key player in cellular senescence, is involved in the process of IS-induced cellular senescence in HUVECs. As shown in Fig. 1B, there were no significant changes in the Sirt1 protein expression, as determined on an immunoblotting analysis, among the IS-treated HUVECs; however, the Sirt1 activity detected using an HDAC colorimetric assay was significantly suppressed at 24 hours (Fig. 1C). It is well known that the Sirt1 activity is dependent on the cellular NAD\(^+\) content. Therefore, in order to assess whether the IS-induced decrease in the Sirt1 activity is caused by NAD\(^+\) depletion, we evaluated the effects of IS on the NAD\(^+\) content using a colorimetric assay. As demonstrated in Fig. 1D, the NAD\(^+\)/NADH ratio decreased significantly from six to 24 hours after IS treatment. Furthermore, we examined the effects of IS on iNampt, the rate-limiting enzyme in the biosynthesis of cellular NAD\(^+\), in order to investigate the mechanism underlying the NAD\(^+\) depletion induced by IS. As shown in Fig. 1E, the iNampt activity analyzed using the colorimetric assay, as well as the NAD\(^+\)/NAPH ratio, significantly decreased in a time-dependent manner. Taken together, these results suggest that IS suppresses the Sirt1 activity by decreasing the iNampt activity and cellular NAD\(^+\) contents and that Sirt1 is involved in the process of cellular senescence induced by IS in HUVECs.

**IS-Enhanced Oxidative Stress Impairs the iNampt-NAD\(^+\)-Sirt1 System, Leading to the Acceleration of Cellular Senescence in HUVECs**

Previously, we reported that IS activates NADPH oxidase-4, resulting in the induction of oxidative stress in HUVECs. Since oxidative stress has been demonstrated to cause cellular senescence due to impairment of the iNampt-NAD\(^+\)-Sirt1 system, we examined the effects of apocynin (600 μmol/L), a specific NADPH oxidase inhibitor, on IS-induced cellular senescence in HUVECs. As shown in Fig. 2A, IS induced cellular senescence at 24 hours in a similar pattern to that induced by 200 μmol/L of H\(_2\)O\(_2\), while pretreatment with apocynin for one hour rescued the IS-induced cellular senescence, suggesting that the enhancement of oxidative stress following IS-induced NADPH oxidase-4 activation results in cellular senescence in HUVECs. Furthermore, we examined whether IS impairs the iNampt-NAD\(^+\)-Sirt1 system by increasing oxidative stress in HUVECs using apocynin. As demonstrated in Fig. 2B-(1), there were no significant changes in the total p53 protein expression on the immunoblotting analysis; however, the acetylated p53 protein expression was significantly enhanced by IS at 24 hours, indicating the reduced enzymatic activity of Sirt1 as a protein deacetylase. With respect to the effects of apocynin on the iNampt-NAD\(^+\)-Sirt1 system, as shown in Fig. 2B-(2), 2C and 2D, the addition of apocynin restored the Sirt1 activity, as detected in the HDAC colorimetric assay, as well as the protein expression of acetylated p53 and ameliorated both the NAD\(^+\)/NADH ratio and iNampt activity at 24 hours after IS treatment. Taken together, these results indicate that IS-enhanced oxidative stress impairs the iNampt-NAD\(^+\)-Sirt1 system, thus accelerating cellular senescence in HUVECs.

**IS-Induced Endothelial Senescence is AhR-Dependent**

We recently reported that the mechanism by which IS increases oxidative stress in HUVECs is AhR-dependent. We therefore examined the effects of AhR blockade on cellular senescence. Pretreatment with ANF (10 μmol/L) or CH223191 (10 μmol/L), AhR inhibitors, for one hour rescued the IS-induced cellular senescence in the HUVECs (Fig. 3A). Furthermore, we analyzed whether AhR is involved in the IS-induced impairment of the iNampt-NAD\(^+\)-Sirt1 system using AhR inhibitors. At 24 hours after IS treatment, the addition of either ANF or CH223191 significantly abolished the reduction in the iNampt activity and NAD\(^+\)/NADH ratio, which in turn restored both the Sirt1 activity determined on the HDAC colorimetric assay and the protein expression of acetylated p53 to the control levels (Fig. 3B-(1) (2), 3C and 3D). Taken together, these findings clearly demonstrate that IS-induced endothelial senescence is AhR-dependent.

**The Addition of NAM Improves IS-Induced Senescence-Related Changes in HUVECs**

Furthermore, in order to confirm that cellular NAD\(^+\) depletion by IS plays a role in the pathogenesis of cellular senescence, we evaluated the effects of NAM, a substrate of NAD, on the IS-induced senescence-related changes. As shown in Fig. 4A, 4B-(1) (2) and 4C, the addition of NAM at a concentration of 5 mmol/L, which is often used in many investigations, with beneficial effects in cardiovascular cells, clearly restored the IS-induced changes observed in the NAD\(^+\) content and Sirt1 activity, resulting in the amelioration of cellular senescence. These results therefore demonstrate that the reduction in the NAD\(^+\) content induced by IS deteriorates the Sirt1 activity, thereby leading to the induction of cellular senescence in HUVECs.
Fig. 2. IS-enhanced oxidative stress impairs the iNampt-NAD\(^+\)-Sirt1 system, leading to the acceleration of cellular senescence in HUVECs.

HUVECs were pretreated with or without apo (600 \(\mu\)mol/L) for one hour and then incubated with or without IS (500 \(\mu\)mol/L) for 24 hours. HUVECs treated with \(\text{H}_2\text{O}_2\) (200 \(\mu\)mol/L) for 24 hours were used as a positive control. (A) Cellular senescence was analyzed using SA \(\beta\)-gal staining, and the percentage of SA \(\beta\)-gal-positive cells was scored by counting the number of blue and total cells within two different fields which under a microscope per sample. The values are presented as the mean \(\pm\) standard error of the mean (SEM) of each sample (\(n=3\)). (B) (1) The protein expression of acetylated p53 was analyzed using immunoblotting. (2) The Sirt1 activity was detected using an HDAC colorimetric assay. (C) The NAD\(^+\)/NADH ratio was measured using a colorimetric assay. (D) The iNampt activity was analyzed using a colorimetric assay. The values are presented as the mean \(\pm\) standard error of the mean (SEM) of three independent experiments for B-D (\(n=4\) or 10). \(^*\) \(p<0.05\), \(^{**}\) \(p<0.01\), \(^{***}\) \(p<0.001\) vs. Control; \(^{††}\) \(p<0.01\) vs. IS. IS, indoxyl sulfate; iNampt, intracellular nicotinamide phosphoribosyltransferase; NAD, nicotinamide adenine dinucleotide; Sirt1, sirtuin 1; HUVECs, human umbilical vein endothelial cells; apo, apocynin; SA \(\beta\)-gal, senescence-associated \(\beta\)-galactosidase; HDAC, histone deacetylase.
Fig. 3. IS-induced endothelial senescence is AhR-dependent.

HUVECs pretreated with or without ANF (10 μmol/L) or CH (10 μmol/L) for one hour were incubated with or without IS (500 μmol/L) for 24 hours. (A) Cellular senescence was detected using SA β-gal staining, and the percentage of SA β-gal-positive cells was scored by counting the number of blue and total cells within two different fields observed under a microscope per sample. The values are presented as the mean ± standard error of the mean (SEM) of each sample (n = 3). (B) (1) The protein expression of acetylated p53 was analyzed using immunoblotting. (2) The Sirt1 activity was detected using an HDAC colorimetric assay. (C) The NAD+/NADH ratio was measured using a colorimetric assay. (D) The iNampt activity was analyzed using a colorimetric assay. The values are presented as the mean ± standard error of the mean (SEM) of three independent experiments for B-D (n = 4 or 10). *p < 0.05, †p < 0.01, ‡p < 0.001 vs. Control; ††p < 0.05, †††p < 0.01 vs. IS. IS, indoxyl sulfate; AhR, aryl hydrocarbon receptor; HUVECs, human umbilical vein endothelial cells; ANF, a-naphthoflavone; CH, CH223191; SA β-gal, senescence-associated β-galactosidase; Sirt1, sirtuin 1; HDAC, histone deacetylase; NAD, nicotinamide adenine dinucleotide; iNampt, intracellular nicotinamide phosphoribosyltransferase.
AhR and Endothelial Senescence Induced by IS

Vascular senescence, which is induced by enhanced oxidative stress in association with the progression of renal failure, is believed to be deeply involved in the onset and progression of cardio-renal syndrome. Sirt1 is a key player in cellular senescence, and the decrease in the iNampt activity and cellular NAD$^+$ content induced by oxidative stress has been shown to impair the Sirt1 activity. IS, indoxyl sulfate, was shown to suppress the Sirt1 activity in association with a decrease in the iNampt activity and NAD$^+$ content, resulting in the acceleration of cellular senescence due to oxidative stress and that IS-induced cellular senescence in HUVECs is AhR-dependent. These findings suggest that the IS-AhR pathway plays an important role in the pathogenesis of vascular senescence, which ultimately leads to the development of cardio-renal syndrome.

Discussion

The present study shows for the first time that IS suppresses the Sirt1 activity in association with a decrease in the iNampt activity and NAD$^+$ content, resulting in the acceleration of cellular senescence due to oxidative stress and that IS-induced cellular senescence in HUVECs is AhR-dependent. These findings suggest that the IS-AhR pathway plays an important role in the pathogenesis of vascular senescence, which ultimately leads to the development of cardio-renal syndrome.

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which accumulates as renal dysfunction progresses, enhances oxidative stress in the setting of CKD. Furthermore, the mechanism by which IS enhances oxidative stress has been reported to involve the transportation of IS into the cell via OAT1 and 3, where it subsequently generates superoxide by activating NADPH oxidase in endothelial, renal tubular and glomerular mesangial cells. Although IS is known to induce endothelial senescence due to oxidative stress, no previous reports have investigated whether Sirt1 is involved in the pathogenesis of IS-induced endothelial senescence. In the present study, we therefore examined the effects of IS on the iNampt activity, cellular NAD⁺ content and Sirt1 activity by adding IS to HUVECs and found that IS decreases both the iNampt activity and NAD⁺ content, resulting in the suppression of the Sirt1 activity. Furthermore, we investigated whether the increased oxidative stress induced by IS plays a role in the impairment of the iNampt-NAD⁺-Sirt1 system using an NADPH oxidase inhibitor. Pretreatment with the NADPH oxidase inhibitor clearly restored the iNampt activity, NAD⁺ content and Sirt1 activity, as detected on an HDAC colorimetric assay, as well as the protein expression of acetylated p53 and cellular senescence in the HUVECs. These results suggest, for the first time, that IS-enhanced oxidative stress impairs the iNampt-NAD⁺-Sirt1 system, leading to the acceleration of endothelial senescence. We previously revealed that the mechanism by which IS increases oxidative stress in HUVECs is AhR-dependent. AhR, a ligand-activated nuclear receptor/transcription factor, is known to mediate drug metabolism, thereby promoting both cancer and fetal organ development. Recently, the activation of AhR by its ligands, including TCDD and PCBs, has been demonstrated to cause endothelial dysfunction and inflammation by increasing oxidative stress. We also previously reported that IS-induced AhR activation stimulates the MCP-1 expression via the actions of MAPK/NF-kB in HUVECs, suggesting that endothelial AhR plays an important role in vascular damage, although it is unclear whether AhR is involved in the pathogenesis of cellular senescence in endothelial cells. We therefore examined whether AhR mediates IS-induced endothelial senescence using AhR inhibitors. The blockade of AhR by its inhibitors canceled the observed cellular senescence in association with the restoration of the iNampt activity, NAD⁺ content and Sirt1 activity in the HUVECs. These results clearly indicate, for the first time, that IS-induced endothelial senescence is AhR-dependent. Many studies have shown that an increase in the NAD⁺ content brings about beneficial effects on the cardiovascular system. For example, Tong et al. reported that NAM pretreatment increases the cellular NAD⁺ content in cardiomyocytes, thus preventing impairment by hypoxia-induced oxidative stress in these cells. In agreement with these reports, we found that the addition of NAM to IS-treated HUVECs markedly reversed the observed decrease in the NAD⁺ content and Sirt1 activity, thereby ameliorating cellular senescence. These results suggest the possibility that supplementation with NAM may prevent the development of CVD induced by IS in CKD patients. Many dialysis patients exhibit a lack of NAM due to its removal during dialysis and limits on the intake of foods containing NAM in medical diets. However, the European Best Practice Guidelines (EBPG) on nutrition advise that high doses of NAM should be prescribed with great caution in dialysis patients when used for the purpose of reducing cardiovascular risks, since there is no evidence that the addition of NAM suppresses cardiovascular events in dialysis patients at the present time.

Based on the fact that IS causes a variety of adverse effects, such as vascular senescence, inflammation and dysfunction, it is important to reduce the circulating IS level and/or block the actions of IS in order to prevent the development of CVD in CKD patients. The use of AST-120 (Kremezin, Kureha Corporation, Tokyo, Japan), a charcoal sorbent, currently accepted to be an effective therapy for CKD, is reported to slow the progression of CKD and delay the initiation of dialysis in humans. The beneficial effects of AST-120 on the vasculature have also been frequently reported. For example, Yu et al. reported that IS-induced endothelial dysfunction can be reversed by AST120 in CKD patients, while Mulelieu et al. demonstrated that treatment with AST-120 prevents cellular senescence and calcification of the aorta induced by IS in renal failure rats. Suggesting that reducing the circulating IS level may be an effective therapy, not only for CKD, but also CVD. In the present study, we found that the IS-AhR pathway induces cellular senescence by increasing oxidative stress in endothelial cells, suggesting that blocking this pathway may prevent IS-induced vascular senescence. However, the molecular mechanisms of AhR-NF-E2-related factor-2 (Nrf2) cross-talk should be taken into account. Yeager et al. reported that AhR plays a role in Nrf2 activation in the liver in mice administered TCDD, and Tsuji et al. demonstrated that the anti-inflammatory effects of ketoconazole, a widely used imidazole antifungal agent, via Nrf2 activation require AhR signaling in normal human epidermal keratino-
cells), suggesting that AhR signaling may be important as a biological defense mechanism. In view of these reports, the complete blockade of the IS-AhR pathway may result in adverse effects in various organs, including the vasculature; therefore, further investigation is required to explore the physiological effects of the AhR signaling pathway on different tissues and cells.

The role of extracellular Nampt (eNampt) in the pathogenesis of cardio-renal syndrome should also be considered. eNampt has been shown to be synthesized and released into the serum by many cell types and variously acts in a paracrine or endocrine manner. In addition, many studies have reported that the serum levels of eNampt are elevated in CKD patients, and Vanholder et al. suggested that the serum IS level is directly correlated with the serum eNampt level. As to the effects of eNampt on the cardiovascular system, eNampt has been demonstrated to induce the production of endothelial nitric oxide synthase (eNOS), resulting in increased NO levels in HUVECs and human coronary artery endothelial cells. Furthermore, eNampt has also been reported to increase the cellular NAD\(^+\) level, thus conferring a higher degree of resistance of cardiomyocytes to oxidative stress under conditions of ischemia reperfusion. These reports suggest the beneficial effects of eNampt on the cardiovascular system; however, there is some controversy regarding the role of eNampt. Liu et al. reported that an increased serum eNampt level plays a role in the development of coronary artery disease, while Malyszko et al. indicated that the serum level of eNampt is positively correlated with that of various circulating inflammation-associated markers, such as IL-6, MCP-1, VCAM-1 and intercellular adhesion molecule-1 (ICAM-1), in humans. As further evidence supporting these reports, eNampt has been shown to induce the activation of NADPH oxidase and the mRNA expression of IL-6, MCP-1, VCAM-1 and ICAM-1 in HUVECs. More recently, Hara et al. demonstrated that eNampt does not contribute to NAD\(^+\) biosynthesis in mice. Given the above findings, eNampt, the level of which is increased in association with the accumulation of IS, may be involved in the pathogenesis of endothelial senescence as well as endothelial inflammation in vivo. Therefore, further studies should be performed in order to clarify the pathophysiological effects of eNampt on the incidence of cardio-renal syndrome.

In summary, we herein demonstrated for the first time that AhR activation by IS accelerates cellular senescence by suppressing the Sirt1 activity in association with a decrease in the NAD\(^+\) content and iNampt activity in endothelial cells. Therefore, in view of the involvement of vascular senescence in the development of CVD, our results suggest that blocking the IS-AhR pathway may provide a novel therapeutic strategy for treating cardio-renal syndrome.

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### Conflicts of Interest
None.

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