The Role of Uremic Toxins in Renal Anemia

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The Role of Uremic Toxins in Renal Anemia

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Abstract

Renal dysfunction causes accumulation of many kinds of compounds in the blood and tissues of patients with chronic kidney disease (CKD), directly or indirectly due to a deficient renal clearance. These compounds that interact negatively with biological functions are called uremic toxins, and approximately 90 compounds have been reported. In particular, protein-bound uremic toxins attract much attention because they are less efficiently removed by hemodialysis and possibly contribute to CKD-associated complications.

Renal anemia is a representative complication associated with CKD. The principal cause of renal anemia is impaired erythropoiesis in the bone marrow due to the inadequate erythropoietin (EPO) production from the kidneys. A decrease in renal interstitial EPO-producing cells due to transformation to a pathologic fibrogenic state associated with renal interstitial fibrosis is considered as a mechanism of the impairment of EPO production. In addition, a recent report demonstrates that a representative protein-bound uremic toxin indoxyl sulfate (IS), although at higher concentrations than the physiological level, suppresses transcriptional activation of hypoxia-inducible factor (HIF), a key transcription factor for hypoxia-induced *EPO* mRNA expression, and subsequent EPO production, suggesting that protein-bound uremic toxins also contribute to the progression of renal anemia by inducing dysfunction of intracellular HIF signaling. However, the mechanism of the impairment of HIF signaling by IS as well as the effects on the EPO production at the physiological concentration are not elucidated yet. Furthermore, there are no investigations regarding the effects of protein-bound uremic toxins other than IS on the EPO production.

In the first part, I have performed *in vitro* and *in vivo* experiments to elucidate the detailed effects of IS at physiological concentrations on the HIF signaling and EPO expression. The results indicate that IS at concentrations similar to the blood levels in CKD patients suppressed the expression of *EPO* mRNA and the transcriptional activation of HIF. Moreover, IS strongly activated the signaling pathway of aryl hydrocarbon receptor (AHR) in both *in vitro* and *in vivo*, and blockade of AHR resulted in abolishment of IS-induced suppression of HIF activation. These findings demonstrate that AHR plays a crucial role in the suppressive effects of IS on hypoxia-induced HIF activation and subsequent EPO production.

In the second part, I have performed *in vitro* experiments to identify the other protein-bound uremic toxins that have a potential to suppress the HIF signaling and EPO expression. The results indicate that indoxyl glucuronide (IG) but not *p*-cresyl sulfate (PCS), phenyl sulfate, 3-indoleacetic acid or hippuric acid, suppressed the expression of *EPO* mRNA induced by hypoxic stimuli. Moreover, IG at concentrations similar to the blood levels in CKD patients suppressed hypoxia-induced transcriptional activation of HIF. As similar to IS, IG also induced the activation of AHR and blockade of AHR abolished the IG-induced inhibition of HIF activation. These results suggest that IG as well as IS is involved in the impairment of HIF signaling in renal anemia.

Collectively, these findings demonstrate that IS and IG contribute to the progression of renal anemia in CKD patients whose blood levels of these compounds increase due to impaired renal excretion. Moreover, this study is the first to elucidate the molecular mechanism by which the activation of AHR plays a crucial role in the suppressive effect of these protein-bound uremic toxins. Hence, protein-bound uremic toxins-induced AHR activation may be one of potential therapeutic targets for treating

renal anemia. These findings warrant further studies to determine the therapeutic effects of the inhibition of AHR.

Abbreviations

AHR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
BFU-E	burst-forming unit-erythroid
CFU-E	colony-forming unit-erythroid
$CoCl_2$	cobalt chloride
CKD	chronic kidney disease
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EPO	erythropoietin
ESA	erythropoiesis stimulating agent
ESRD	end stage renal disease
FBS	fetal bovine serum
GFR	glomerular filtration rate
HA	hippuric acid
HIF	hypoxia-inducible factor
HRE	hypoxia responsive element
IAA	3-indoleacetic acid
IG	indoxyl glucuronide
IS	indoxyl sulfate
MEM	minimal essential medium
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

PBS	phosphate-buffered saline
PCS	*p*-cresyl sulfate
PHD	prolyl-4 hydroxylase
PhS	phenyl sulfate
PVDF	polyvinylidene difluoride
REPs	renal EPO-producing cells
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
VHL	von Hippel–Lindau
XRE	xenobiotic response element

General Introduction

1. Function of kidney

The kidneys have a central role in the excretion of xenobiotics and its metabolites to the urine. In the renal cortex which is the outer portion of the kidney, numerous compounds are filtrated from the blood by the glomerular into the primary urine. Then, reabsorption of glucose, electrolytes, amino acids, and H_2O from the primary urine are processed in the proximal renal tubular. The renal tubular also has the function to excrete compounds unable to be filtrated in glomerular to the urine. After further reabsorption of electrolytes and H_2O, the urine is collected and excreted into the urinary duct through the renal medulla which is the inner portion of the kidney.

The kidneys also have functions as an endocrine organ, and secrete a variety of hormones and hormone-related molecules, including renin, activated vitamin D, and erythropoietin (EPO). Renin is a key enzyme for the control of blood pressure by regulating angiotensin and aldosterone levels. Activated vitamin D promotes intestinal absorption of calcium and the renal reabsorption of phosphate, which regulates calcium and phosphate levels in the blood. EPO is released from renal interstitial fibroblast-like cells in response to hypoxia in the renal circulation and stimulates erythropoiesis in the bone marrow.

2. Chronic kidney disease

Chronic kidney disease (CKD) is defined as abnormalities of kidney structure or function, persisting for over 3 months, with indication of kidney damage in urinalysis, image diagnosis and histopathology or with a decrease in glomerular filtration rate (GFR) to less than 60 mL/min/1.73m^2 (normally 90 mL/min/1.73m^2 or more in healthy subject)[1)]. Diabetes, chronic glomerulonephritis, and renal sclerosis are major primary

diseases causing CKD. Under these primary diseases, hyperglycemia, inflammation, and renal atherosclerosis due to sustained hypertension cause renal glomerular and tubular injury, which in turn induces renal fibrosis. The severity of CKD is classified as stage 1 to 5 by the value of GFR (Table 2). In stage 5 which is called end stage renal disease (ESRD), hemodialysis for the entire lifetime or kidney transplantation is needed for the treatment of CKD.

The estimated number of CKD patients is approximately 13.3 million in Japan and it increases every year. In particular, the number of ESRD patients with dialysis reached 330 thousands in 2016 and the increase in medical expenses related to dialysis treatment is a major problem [2]. Furthermore, several kinds of complications such as cardiovascular disease, anemia, mineral and bone disorders, and central and peripheral nerve disorders are frequently observed in CKD patients. Therefore, the treatment of CKD must have attention to not only renal function but also these complications.

3. Uremic toxins and the biological effects

Many kinds of compounds accumulate in the blood and tissues of patients with CKD, directly or indirectly due to a deficient renal clearance. These compounds that interact negatively with biological functions are called uremic toxins or uremic retention solutes, and more than 90 compounds have been reported [3, 4]. Based on the molecular weight and protein binding properties, these uremic toxins are classified into the following three groups: freely water-soluble low molecular weight molecules, protein-bound molecules and middle molecules (Table 1). In particular, protein-bound uremic toxins attract much attention because they are less efficiently removed by hemodialysis [5] and possibly have a role in CKD-associated complications [6, 7].

Indoxyl sulfate (IS), a metabolite of tryptophan, is a representative protein-bound uremic toxin. Food-derived tryptophan is converted to indole by intestinal microflora, and thereafter in the liver, indole is metabolized to IS through oxidization to indoxyl by cytochrome P450 enzymes and conjugation with sulfate by sulfotransferases [8, 9]. IS then enters into the bloodstream and is finally excreted into urine by mainly tubular secretion in the kidneys (Figure 1). Many studies demonstrate that IS contributes to the progression of CKD by inducing oxidative stress in renal tubular cells [10, 11, 12]. IS has also been reported to induce oxidative stress in vascular smooth muscle cells [13, 14, 15] and endothelial cells [16], suggesting its involvement in cardiovascular diseases associated with CKD [17, 18].

The biological effects of other protein-bound uremic toxins are also investigated in recent years. As similar to IS, *p*-cresyl sulfate (PCS), a metabolite of tyrosine, has been reported to be involved in the progression of CKD [19] and cardiovascular diseases associated with CKD [18, 20] through inducing oxidative stress. In addition, 3-indoleacetic acid and hippuric acid have been reported to exacerbate the progression of CKD in animal experiments [21].

Table 1. *Major Uremic toxins.*

Group	Uremic toxin	Molecular weight
Freely water-soluble low molecular weight molecules	1-Methyladenosine	281
	Creatinine	113
	Guanidine	59
	Guanidinoacetic acid	117
	Mannitol	182
	Sorbitol	182
	Thymine	126
	Uracil	112
	Uric acid	168
	Xanthine	152
Protein-bound molecules	Acrolein	56
	Hippuric acid	179
	Homocysteine	135
	3-Indoleacetic acid	175
	Indoxyl sulfate	213
	Indoxyl glucuronide	309
	Kinurenine	208
	Melatonin	126
	p-Cresyl sulfate	188
	Pentosidine	342
	Phenyl sulfate	175
	Polyamines	88-202
Middle molecules	Adrenomedullin	5729
	β_2-Microglobulin	11818
	β-Endorphin	3465
	Cystatin C	13300
	Endothelin	4283
	Hyaluronic acid	25000
	Interleukin-6	24500
	Leptin	16000
	Parathyroid hormone	9225
	Tumor necrosis factor-α	26000

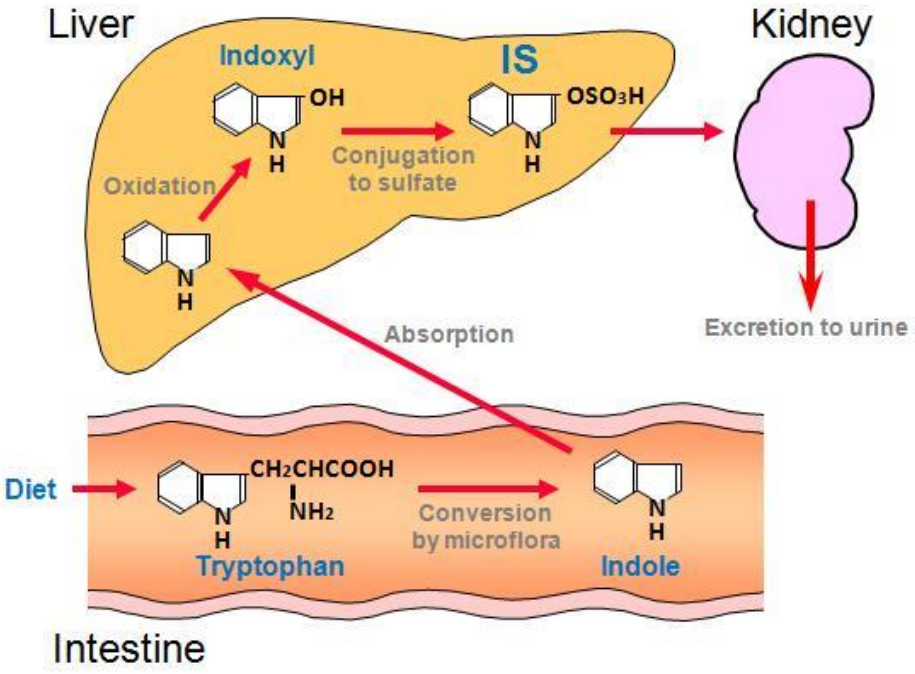

Figure 1. *Metabolic production and excretion of IS.*

4. Renal anemia

Anemia is a representative complication associated with CKD, which is called renal anemia based on the mechanism related to the renal damage. Similar to common anemia, patients with renal anemia present decreases in hemoglobin concentration and the number of erythrocytes (or red blood cells) in peripheral blood. As shown in Table 2, renal anemia occurs apparently in patients over CKD stage 3 and more than 50% of patients with CKD stage 4 and 5 are suffered from that [22].

A clinical study demonstrates that CKD patients with lower hemoglobin concentration have higher risk for the progression to ESRD (CKD stage 5)[23]. Furthermore, an observational study that monitored the survival of CKD patients, not yet on dialysis, for approximately 3 years after measurement of hemoglobin concentration indicates that patients with lower hemoglobin concentration show lower survival rate (Figure 2)[24]. Thus, the appropriate control of renal anemia is important for not only the improvement of quality of life but also the prognosis of CKD and survival.

Table 2. *Prevalence of anemia by CKD stage.*

This table is modified from Inker *et al.*, 2011 [22].

CKD stage	GFR (mL/min/1.73 m^2)	Prevalence of anemia
1	≥90	4.0%
2	60-89	4.7%
3a	45-59	12.3%
3b	30-44	22.7%
4	15-29	51.5% (stage 4&5)
5	<15	

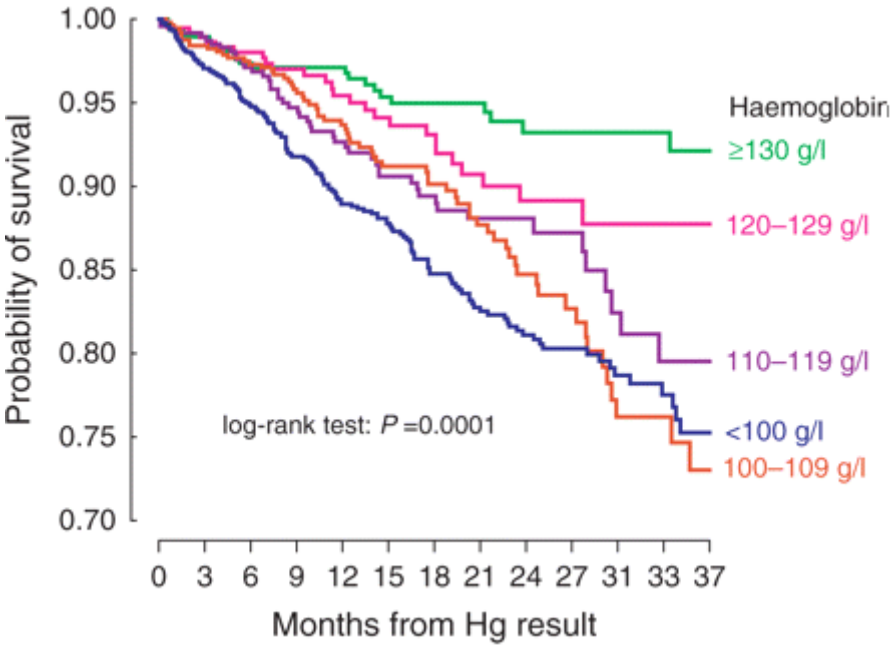

Figure 2. *Survival of CKD patients by hemoglobin (Hg) concentration.*

This figure is modified from Levin *et al.*, 2006 [24].

5. Life cycle of erythrocytes and EPO-mediated erythropoiesis

Erythrocytes have a role to deliver oxygen from lungs to cells of whole body through the blood stream. The physiological life span of human erythrocytes is 100-120 days, and approximately 1% of the cells are renewed every day. The production of erythrocytes (erythropoiesis) mainly takes place in the bone marrow and is facilitated when a decrease of erythrocyte occurs due to bleeding, hemolysis or other causes. EPO is the most essential hormone among the process of differentiation from hematopoietic stem cells to erythrocytes (Figure 3). EPO stimulates cell division, proliferation and differentiation of erythrocyte progenitor cells such as burst-forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E) and erythroblasts. In particular, CFU-E strongly expresses EPO receptors and is the most strongly affected by EPO. Erythroblasts undergo enucleation in the last step of erythropoiesis, and circulate in the blood stream. At the end of the lifespan, erythrocytes are removed and destroyed by macrophages of the mononuclear phagocyte system (also called reticuloendothelial system) mainly in the spleen.

EPO is principally produced from hepatocytes in the liver during fetal state [25]. On the other hand, after birth, EPO-producing organ switched from liver to kidneys and hepatic EPO production is to be supportive (but still produces small amount of EPO in adult). Although the source of EPO in the kidneys has been unclear for many years, recent studies have identified the renal interstitial fibroblast-like cells as EPO producing cells [26, 27]. The transcription of *EPO* mRNA is mediated by hypoxia-inducible factor (HIF), a heterodimeric complex composed of HIF-α subunits (such as HIF-1α and HIF-2α) and aryl hydrocarbon receptor nuclear translocator (ARNT; also called HIF-1β). The expression of HIF-α subunits is strictly controlled by cellular oxygen molecules.

Under normoxia, oxygen-dependent HIF prolyl-4 hydroxylase (PHD) hydroxylates the proline residues of HIF-α subunits, which in turn triggers recruitment of the von Hippel–Lindau (VHL) tumor suppressor protein, a component of E3 ubiquitin ligase complex, and facilitates proteasomal degradation of HIF-α subunits [28, 29]. On the other hand, under hypoxia, HIF-α subunits escape from the degradation pathway, consequently accelerating nuclear accumulation of HIF-α subunits and dimerization with ARNT. This heterodimeric complex binds to hypoxia responsive element (HRE) in the promoter region, and promotes the expression of target genes including *EPO* (Figure 4). The disruption of EPO-producing mechanism induces anemia (described in the following section) or polycythemia. For example, the impairment of HIF degradation pathway due to gene mutations of *VHL* or *PHD* causes hyperproduction of EPO and subsequent polycythemia in human [30, 31].

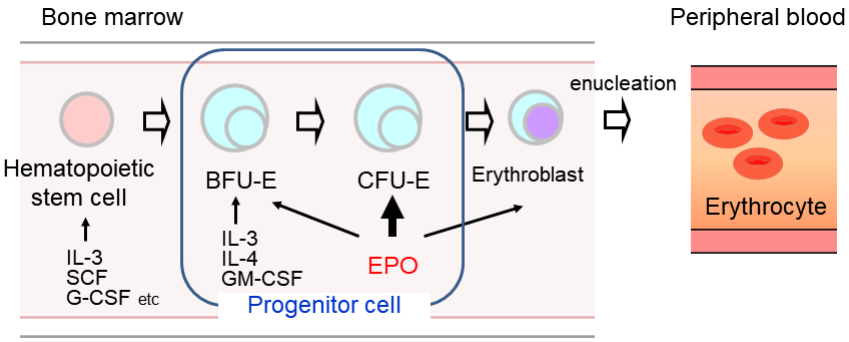

Figure 3. *Differentiation of hematopoietic stem cells into erythrocytes.*

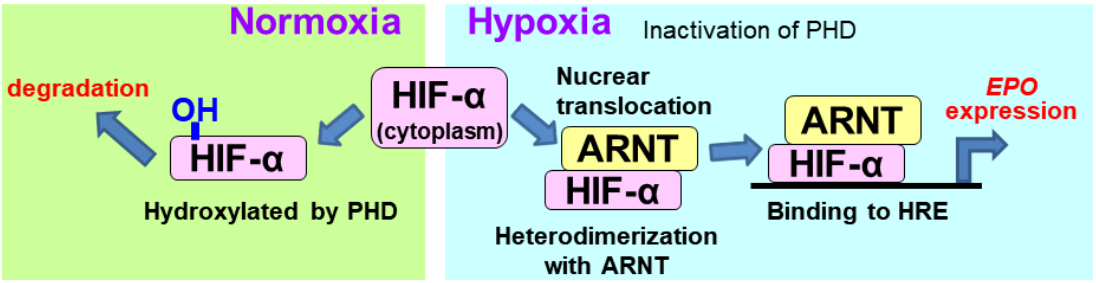

Figure 4. *HIF-mediated EPO expression.*

6. Causes of renal anemia

The principal cause of renal anemia is impaired erythropoiesis in the bone marrow due to the inadequate EPO production from EPO-producing cells in the renal interstitium. The following mechanisms are considered as causes of the impairment of EPO production. The progression of renal interstitial fibrosis associated with renal tissue damage induces transformation of renal interstitial EPO-producing cells to a pathologic fibrogenic state and leads to loss of EPO-producing ability, and the inflammatory microenvironment is suggested as one of the possible causes (Figure 5) [32, 33]. Moreover, Chiang *et al.* [34] have reported that IS, at higher concentrations than the physiological level, suppresses hypoxia-induced HIF activation and subsequent EPO production, suggesting that protein-bound uremic toxins also contribute to the progression of renal anemia by inducing dysfunction of intracellular HIF signaling.

In addition to the impairment of EPO production as the main mechanism of renal anemia, other causes are also known. The erythrocyte life span of dialysis patients (ESRD patients) has been reported to be shortened to 73 days compared with heathy subjects (115 days)[35], indicating that destruction of erythrocytes due to intravascular or extravascular hemolysis could be accelerated in dialysis patients. Furthermore, bone marrow cell culture experiments revealed that inflammatory cytokines in the serum of dialysis patients inhibited the differentiation of erythrocyte progenitors [36, 37], suggesting the suppression of responsiveness of erythrocyte progenitors to growth factors like EPO in dialysis patients.

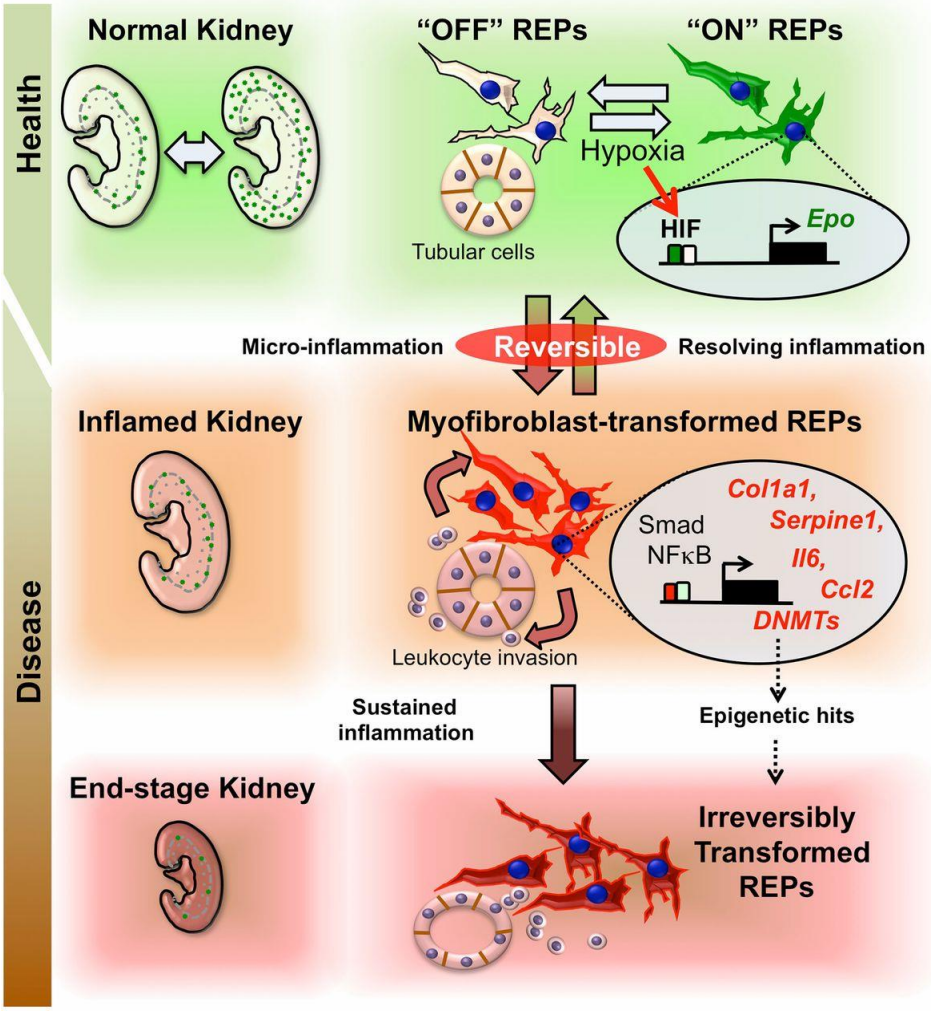

Figure 5. *Phenotypic changes of renal EPO-producing cells (REPs) in health and disease.*

In healthy conditions, most REPs are in "OFF" state, and in response to hypoxic stimuli, REPs turn to "ON" state and start to produce EPO. In injured kidneys, microinflammation and other stresses transform REPs into myofibroblasts, and myofibroblast-transformed REPs lose EPO-producing potential and actively contribute to renal fibrosis. By eliminating the inflammatory signals, myofibroblast-transformed REPs restart producing EPO, regaining their physiologic phenotypes.

This figure is reprinted from Souma *et al.*, 2013 [32].

7. Objective and composition of the thesis

Renal inflammatory microenvironment-induced transformation of EPO-producing cells and protein-bound uremic toxin-induced dysfunction of intracellular HIF signaling in EPO-producing cells are possibly involved in the onset and progression of renal anemia. However, there are no investigations regarding the effects of protein-bound uremic toxins other than IS on the HIF signaling and EPO production. Furthermore, the mechanism of the impairment of HIF signaling by IS as well as the effects on the EPO production at the physiological concentration are not elucidated yet. Elucidation of these subjects will contribute not only to the understanding of the pathophysiological mechanisms of renal anemia but also to its prevention and treatment. For this reason, this thesis describes the following two main subjects:

1. Effect of indoxyl sulfate on hypoxia-induced EPO expression in HepG2 cells and rats (Part I).
2. Effect of uremic toxins other than indoxyl sulfate on hypoxia-induced EPO expression in HepG2 cells (Part II).

In the first part, I performed *in vitro* and *in vivo* experiments to elucidate the detailed effects of IS at physiological concentrations on the HIF signaling and EPO expression, and demonstrated the possible molecular mechanism of the impairment of HIF signaling by IS.

In the second part, I performed *in vitro* experiments to identify the other protein-bound uremic toxins that have a potential to suppress the HIF signaling and EPO expression.

PART I **Effect of indoxyl sulfate on hypoxia-induced erythropoietin expression in HepG2 cells and rats**

Abstract

Indoxyl sulfate (IS) is a representative protein-bound uremic toxin that accumulates in the blood of patients with chronic kidney disease (CKD). A recent report indicates that IS suppresses hypoxia inducible factor (HIF)-dependent erythropoietin (EPO) production, suggesting that IS could contribute to the pathogenesis of renal anemia which occurs due to inadequate EPO production from the kidneys in CKD patients. In this part, I provide evidence that aryl hydrocarbon receptor (AHR) mediates IS-induced suppression of HIF transcriptional activation and subsequent EPO production. In EPO-producing HepG2 cells, IS at clinically relevant concentrations in CKD patients suppressed hypoxia- or cobalt chloride-induced *EPO* mRNA expression and HIF transcriptional activation. IS also induced activation of AHR signaling pathway, and AHR blockade resulted in inhibition of IS-induced suppression of HIF activation. The HIF transcription factor is a heterodimeric complex composed of HIF-α subunits (HIF-1α and HIF-2α) and AHR nuclear translocator (ARNT). IS suppressed nuclear accumulation of the HIF-α–ARNT complex accompanied by an increase of the AHR–ARNT complex in the nucleus, suggesting the involvement of interactions among AHR, HIF-α and ARNT in the suppression mechanism. In rats, oral administration of indole, a metabolic precursor of IS, inhibited bleeding-induced physiological increase in renal *EPO* mRNA expression and plasma EPO concentration. Oral administration of indole also strongly induced the activation of AHR signaling pathway in the liver and renal cortex tissues. Taken together, this study is the first to elucidate the detailed molecular mechanism by which AHR plays a crucial role in the suppression of HIF activation by IS.

Introduction

The principal cause of renal anemia is impaired erythropoiesis in the bone marrow due to the inadequate EPO production from the kidneys. As one of the mechanisms, IS, a representative uremic toxin, has been reported to suppress hypoxia-induced transcriptional activation of HIF and subsequent EPO production [34]. This report provides important knowledge for the elucidation of the biological effects of IS in renal anemia; however, the effects at physiological concentration in CKD patients are not investigated. Furthermore, the molecular mechanism by which IS suppresses HIF activation remains entirely unclear.

Regarding the intracellular target molecule of IS, *in vitro* experiments reported by Schroeder *et al.* revealed that IS was an endogenous ligand for a ligand-activated transcription factor AHR, also called the dioxin receptor [38]. The AHR normally exists in the cytosol in an inactive form and translocates into the nucleus upon binding ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In the nucleus, the AHR dimerizes with ARNT, binds to xenobiotic response element (XRE) in the promoter region, and promotes the expression of several genes including *CYP1A1*, a subfamily of cytochrome P450 enzymes. Investigations of the AHR have focused on its role in the toxic effects of dioxin-like environmental pollutants for many years [39]. In recent years, however, the AHR is known to play a role in fundamental and important physiological functions such as immune function[40], stem cell regulation [41, 42], and carcinogenesis [43]. These findings prompted me to investigate the involvement of the AHR in the biological effects of IS.

In the present study, I investigated the effects of IS at physiological concentrations on the HIF signaling and EPO expression by cell culture experiments

and animal experiments using model rats showing elevated endogenous EPO production by bleeding. In the cell culture experiments, the human hepatoma cell line HepG2 were used because the cell line is derived from the liver which is one of the source tissues of EPO *in vivo* and retains the ability to produce EPO in response to hypoxic stimuli induced by hypoxic culture or treatment with hypoxia mimicking agents such as cobalt chloride [44]. Furthermore, I investigated to determine whether AHR contributes to the suppressive effect of IS on HIF activation.

Materials and Methods

1. *In vivo* experiments in rats

All experimental procedures were approved by the Committee of Ethics on Animal Experiments at Kureha Corporation (Tokyo, Japan), and conducted in accordance with the guidelines of Kureha Corporation. Seven week-old male SD (Crl:CD) rats that weighed 210 to 260 g were obtained from Japan Charles River (Kanagawa, Japan). Rats were housed in polycarbonate cages with free access to water and standard diet in an animal room under controlled illumination (12-hour light/dark cycle), temperature (22 ± 2ºC) and humidity (55 ± 10%). After acclimatization for at least 6 days, the rats were fasted for approximately 20 hours before the beginning of experiments. In the bleeding experiments, 1 mL of blood per 100 g body weight was drawn from the cervical vein of rats under inhaled isoflurane anesthesia for the induction of physiological EPO production. After recovery from anesthesia, the rats were administrated indole (Wako Pure Chemical Industries, Osaka, Japan) suspended in 0.5% methylcellulose solution (Wako Pure Chemical Industries) by oral gavage at a dose of 100 mg/kg body weight to increase the concentration of IS in the blood. Thereafter, blood sampling was performed 3, 9 and 24 hours after indole administration and rats were euthanized after the last blood sampling. For the analysis of the expression of *Epo* mRNA, rats were euthanized 9 hours after indole administration. Soon after euthanasia, the liver and renal cortex tissues were collected, snap frozen in liquid nitrogen and stored at -80ºC until RNA isolation. In the experiments on *Cyp1a1* mRNA expression and AHR protein analysis, healthy rats were administrated with a single oral dose of indole (100 mg/kg) as described above and euthanized at 3, 6 or 9 hours later. The liver and renal cortex tissues were collected, frozen in liquid nitrogen

and stored at -80ºC until further analyses.

2. Measurement of EPO and IS concentrations in rat plasma

EPO concentrations in rat plasma were measured using a Mouse/Rat EPO Serum/Plasma kit (MA6000; Meso Scale Discovery, Rockville, MD, USA) according to the manufacturer's instructions. Plasma IS concentrations were measured by liquid chromatography/electrospray ionization-tandem mass spectrometry (API 4000 LC/MS/MS System; Takara Bio, Shiga, Japan) as described by Kikuchi *et al* [45].

3. Cell culture

The human hepatoma cell line HepG2 was obtained from DS Pharma Biomedical (Osaka, Japan). The culture medium was Minimal Essential Medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 1% non-essential amino acid solution (Sigma-Aldrich), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Tokyo, Japan), except in transfection experiments in which penicillin/streptomycin-free medium was used. Cells were cultured at 37°C in a 5% CO_2 humidified incubator unless otherwise indicated. For induction of cellular hypoxic response, HepG2 cells were treated with a hypoxia mimicking agent cobalt chloride (Wako Pure Chemical Industries) at 50 μM. Alternatively, cells were cultured under hypoxic condition using an AnaeroPack System (Mitsubishi Gas Chemical, Tokyo, Japan) for periods indicated in the figure legends. This system is able to deplete the oxygen concentration to less than 1% in 1 hour and providing a 5% CO_2 atmosphere without changing medium pH [46, 47]. IS obtained from Alfa Aesar (Ward Hill, MA,

USA) was added 1 hour prior to cobalt chloride treatment or immediately before hypoxic culture. In some experiments, the potent AHR agonist TCDD (Cambridge Isotope Laboratories, Tewksbury, MA, USA) dissolved in dimethyl sulfoxide (DMSO) was added instead of IS. The final concentration of DMSO in the medium was set to less than 0.2% in all experiments.

4. Cell viability assay

HepG2 cells (1×10^4/well) were seeded into 96-well plates and cultured overnight. Cells were then treated with IS (0, 500, 1500 or 5000 μM) for 24 hours in the presence or absence of cobalt chloride. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Dojindo Laboratories, Kumamoto, Japan) was added at the end of IS treatment, and cells were further cultured for 1 hour. The formazan crystals were dissolved in DMSO and absorbance at 570 nm was determined using a microplate reader.

5. RNA isolation from HepG2 cells and rat tissues and quantitative real-time RT-PCR

HepG2 cells (2.5×10^5/well) were seeded into 12-well plates and cultured overnight. The growth medium was replaced with the medium containing 0.1% FBS and cells were further cultured overnight under serum starvation. Serum-starved cells were treated with IS and cultured with cobalt chloride or under hypoxic condition as described above. In some experiments, the AHR antagonist CH-223191 (Sigma-Aldrich) dissolved in DMSO was added at the same time of IS treatment. Total RNA was extracted from cultured cells or the liver and renal cortex tissues of rats using

an Illustra RNA Spin mini kit (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. Total RNA (250 ng for cells and 500 ng for tissues in 10 μL reaction volume) was reverse transcribed with random hexamer primers using a PrimeScript RT master mix (Takara Bio). Quantitative real-time RT-PCR was then performed with fivefold-diluted cDNA using a KAPA SYBR fast qPCR kit (Nippon Genetics, Tokyo, Japan) and gene specific primer pairs (35-40 cycles at 95ºC for 3 sec, 60ºC for 30 sec). The sequences of primers used are listed in Table 3. Values for the mRNA were normalized to the expression of *HPRT* mRNA for human genes and *Gapdh* mRNA for rat genes, respectively. Analyses were conducted with a Thermal Cycler Dice Real Time System (Takara Bio).

6. Measurement of HIF transcriptional activity by luciferase reporter gene assay

The luciferase reporter plasmid for HRE (pGL4.42 [*luc2P*/HRE/Hygro] Vector) and control plasmid (pGL4.74 [*hRluc*/TK] Vector) were obtained from Promega (Madison, WI, USA). After seeding into 96-well plate (1×10^4/well) and overnight culture, HepG2 cells were co-transfected with both the HRE-luciferase and control plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, Tokyo, Japan) and cultured for 24 hours. Cells were then treated with IS and cultured with cobalt chloride or under hypoxic condition for periods indicated in the figure legends. Cells were washed with ice-cold phosphate-buffered saline (PBS), and cellular luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega) with a Lumat LB9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). The HIF transcriptional activity (HRE-luciferase activity) is presented as the

ratio of *Photinus pyralis* luciferase activity derived from HRE-luciferase plasmid / *Renillareniformis* luciferase activity derived from control plasmid.

In some experiments, the AHR antagonist CH-223191 dissolved in DMSO was added at the same time of IS treatment. In experiments using siRNA for AHR, cells were transfected simultaneously with the luciferase reporter plasmids and AHR siRNA (ON-TARGETplus siRNA, human *AHR*, Cat. J-004990-07) or non-targeting siRNA (ON-TARGETplus Control siRNA, #1, Cat. D-001810-01-05; both from Thermo Fisher Scientific, Kanagawa, Japan). For the assessment of the knockdown effect of the siRNAs, cells were harvested at the start of IS treatment, lysed in 1× reducing sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 5% 2-mercaptoethanol), and analyzed for AHR protein expression by immunoblot as described below.

7. Nuclear protein extraction from HepG2 cells and rat tissues

HepG2 cells (7.5×10^6/dish) were seeded into 10-cm dishes and cultured overnight. After treatment with IS and cobalt chloride for periods indicated in the figure legends, cells were harvested. Nuclear proteins were extracted from the cells or rat liver and renal cortex tissues using NE-PER Nuclear and Cytoplasmic Extraction Reagents supplemented with Halt Protease Inhibitor Cocktail with EDTA (both from Thermo Fisher Scientific) according to the manufacturer's instructions. Protein concentrations of the nuclear protein extracts were determined using a BCA protein assay kit (Thermo Fisher Scientific) with bovine serum albumin as standard.

8. Immunoprecipitation of ARNT-binding nuclear proteins

Equal amounts (100 μg) of nuclear protein extracts of HepG2 cells were processed by immunoprecipitation procedures using a Pierce Classic IP kit (Thermo Fisher Scientific) with anti-HIF-1β/ARNT (D28F3) XP Rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Proteins bound to the antibody were then eluted with an acid-based buffer.

9. Immunoblot analysis

Cell lysates, nuclear protein extracts and immunoprecipitated nuclear proteins of HepG2 cells and nuclear protein extracts of rat tissues were mixed with reducing SDS sample buffer and heated for 3 min at 100°C. The samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (GE Healthcare). The membranes were blocked with 3% skim milk solution and treated with one of the following primary antibodies: anti-AHR mouse monoclonal antibody (A-3; Santa Cruz Biotechnology, Dallas, TX, USA), anti-HIF-1α rabbit polyclonal antibody, anti-HIF-2α rabbit monoclonal antibody, anti-HIF-1β/ARNT rabbit polyclonal antibody (all from Cell Signaling Technology). As loading controls for cell lysates and nuclear protein extracts, anti-α-tubulin rabbit polyclonal antibody and anti-Lamin B1 rabbit polyclonal antibody (both from Medical & Biological Laboratories, Aichi, Japan) were also used as primary antibody. After treatment with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), signals were visualized using an ECL prime detection kit (GE Healthcare) and a luminescence imager Light-Capture AE-6981 (Atto, Tokyo, Japan).

10. Statistical analysis

Two-tailed unpaired *t* test was performed for single comparisons using Excel 2010 (Microsoft, Tokyo, Japan). For multiple comparisons, Dunnett test or Tukey-Kramer HSD test was performed using JMP (ver. 10.0.0, SAS Institute, Cary, NC, USA). Differences were considered statistically significant for *P* values less than 0.05.

Results

1. IS suppresses hypoxia-induced increase of *EPO* mRNA expression in HepG2 cells

IS had no influence on viability of HepG2 cells at concentrations up to 1500 μM, both in the presence and absence of cobalt chloride (data not shown). Therefore, the highest concentration of IS was set at 1500 μM in subsequent experiments.

In the absence of hypoxic stimulation, IS had no effect on *EPO* mRNA expression up to 1500 μM (Figure 6A). Under hypoxic conditions (O_2<1%), the expression of *EPO* mRNA increased significantly by 2.8-fold and this increase was significantly suppressed by IS at 100 μM or higher (Figure 6B). Similarly, the increase of *EPO* mRNA expression induced by treatment with cobalt chloride was suppressed by IS at 100 μM or higher (Figure 6C).

2. Oral administration of indole causes elevation of plasma IS concentration and suppresses bleeding-induced EPO production in rats

In healthy rats, plasma IS concentration elevated markedly by a single oral administration of indole at 100 mg/kg (5 ± 1 μM in vehicle-treated rats; 127 ± 23, 52 ± 19 and 29 ± 9 μM at 3, 6 and 9 hours, respectively, after administration in indole-treated rats).

In the bleeding experiments in which 1 mL of blood per 100 g body weight was drawn from each rat, mean plasma IS concentration elevated up to 140 μM by a single oral administration of indole at 100 mg/kg, whereas IS concentration was less than 10 μM in the vehicle group throughout the experimental periods (Figure 7A). Plasma

EPO concentration elevated gradually over time after blood withdrawal in the vehicle group, but the elevation was significantly suppressed by administration of indole (Figure 7B). Blood withdrawal also induced the expression of *Epo* mRNA in the renal cortex but not in the liver, and the induction was significantly suppressed by administration of indole (Figure 7C).

3. IS suppresses hypoxia-induced HIF activation in HepG2 cells

Transcriptional activity of HIF was assayed by measuring HRE-luciferase activity in HepG2 cells. In the absence of hypoxic stimulation, IS had no influence on HIF transcriptional activity even at 500 μM (Figure 8A). Hypoxic culture (O_2<1%) caused an increase in HIF transcriptional activity by 6.50-fold and this increase was significantly suppressed by IS at 100 μM or higher (Figure 8B). Moreover, in the case of cobalt chloride treatment, IS significantly suppressed the increase in HIF transcriptional activity at 20 μM or higher (Figure 8C). Since transcriptional activation of HIF is induced by the accumulation of HIF-α subunits in the nucleus, the amounts of HIF-1α and HIF-2α in nuclear protein extracts were measured. Cobalt chloride treatment caused nuclear accumulation of both HIF-1α and HIF-2α proteins, and this accumulation was apparently suppressed by IS (Figure 8D).

To examine the possibility that the IS-induced suppression of HIF activation is caused by the inhibition of endogenous expression of HIF-α subunits, the effect of IS on mRNA expression of HIF-α subunits was investigated. IS had no effect on *HIF2α* mRNA expression up to 500 μM in the presence or absence of cobalt chloride (data not shown). IS also had no effect on *HIF1α* mRNA expression in the absence of cobalt chloride, although IS induced a slight increase in *HIF1α* mRNA expression only at

500 μM in the presence of cobalt chloride (Figure 8E). Therefore, IS did not decrease *HIF1α* or *HIF2α* mRNA expression. Consequently, IS suppressed nuclear accumulation of HIF-α subunits by mechanisms other than inhibition of endogenous expression.

4. Inactivation of AHR inhibits the IS-induced suppression of HIF activation and *EPO* mRNA expression in HepG2 cells

In the absence of hypoxic stimulation, HIF transcriptional activity assayed by measuring HRE-luciferase activity was not affected by treatment with IS, an AHR antagonist CH-223191, or both (Figure 9A). The increase in HIF transcriptional activity induced by hypoxic culture (O_2<1%) was significantly suppressed by IS (100 μM), and IS-induced suppression was completely inhibited by co-treatment with CH-223191 (1 μM), while CH-223191 alone had no influence on hypoxia-induced HIF activation (Figure 9B). Similarly, the increase in HIF transcriptional activity induced by cobalt chloride was significantly suppressed by IS (100 μM) and this suppression was inhibited by co-treatment with CH-223191 in a concentration-dependent manner (Figure 9C). In the experiments using siRNA, transient transfection of AHR-siRNA inhibited cellular expression of AHR protein, whereas transfection of non-targeting siRNA had no effect (Figure 9D). Cobalt chloride treatment induced significant increases in HIF transcriptional activity in all cells, and this increase was suppressed by IS (100 μM) in non-targeting siRNA–transfected cells but not in AHR-siRNA–transfected cells (Figure 9E). Hence, transfection of AHR-siRNA inhibited the suppression of cobalt chloride-induced HIF activation by IS.

Since the HRE-luciferase assay measures HIF transcriptional activation but not target gene expression, the effect of AHR inactivation on the suppression of *EPO*

mRNA expression by IS was further investigated. The increase in *EPO* mRNA expression induced by hypoxic culture (O_2<1%) was significantly suppressed by IS (500 μM), and this suppression was inhibited by co-treatment with CH-223191 (1 μM), whereas CH-223191 alone had no effect on hypoxia-induced *EPO* mRNA expression (Figure 9F).

5. The potent AHR agonist TCDD suppresses hypoxia-induced HIF activation in HepG2 cells

As shown in Figure 10, treatment with TCDD and/or CH-223191 had no effect on HIF transcriptional activity. In contrast, cobalt chloride-induced increase in HIF transcriptional activity was significantly suppressed by treatment with TCDD (10 nM) and this suppression was completely inhibited by co-treatment with CH-223191. Moreover, cobalt chloride-induced nuclear accumulation of HIF-α subunits was suppressed by TCDD as well as by IS (Figure 8D).

6. IS activates AHR signaling pathway in both HepG2 cells and rats

In *in vitro* experiments using HepG2 cells, IS strongly induced the expression of *CYP1A1* mRNA, one of the major genes regulated by AHR, by 50-fold even at 100 μM, the lowest concentration investigated (Figure 11A). Moreover, the amount of AHR in nuclear protein extracts apparently increased by IS in a concentration-dependent manner (Figure 11B).

In *in vivo* experiments using healthy rats, single oral administration of indole at a dose of 100 mg/kg that caused marked increases in plasma IS concentration (as described above) induced significant increase in *Cyp1a1* mRNA expression in both the

liver and renal cortex tissues compared with vehicle-treated control animals; particularly in the renal cortex tissue, the increase reached 726-fold at 3 hours after indole administration, and still remained 16-fold at 9 hours although there was no statistical significance (Figure 12A). In the liver, the amount of AHR in nuclear protein extracts increased significantly by administration of indole (Figure 12B). Meanwhile, in the renal cortex tissue, AHR was undetectable in the nuclear protein extracts of all samples, due to the small amount of this protein (data not shown).

7. IS increases AHR–ARNT complex but decreases HIF-α–ARNT complex in the nucleus of cobalt chloride-treated HepG2 cells

To evaluate the amounts of both AHR–ARNT and HIF-α–ARNT complexes in the nucleus, ARNT-binding proteins in the nuclear protein extracts of cobalt chloride-treated HepG2 cells were immunoprecipitated using anti-ARNT antibody and the expressions of AHR, HIF-1α, HIF-2α and ARNT were detected by immunoblot. IS treatment increased the amount of AHR bound to ARNT in a concentration-dependent manner. By contrast, the amounts of HIF-α subunits (HIF-1α and HIF-2α) bound to ARNT decreased in inverse proportion to the increase in amount of the AHR–ARNT complex (Figure 13).

Discussion

Renal anemia is a representative complication associated with CKD patients, which is mainly caused by impaired erythropoiesis in the bone marrow due to the inadequate EPO production from the damaged kidneys. Renal dysfunction also causes accumulation of uremic toxins due to reduced excretion from the kidneys. Several reports demonstrate that uremic toxins could contribute to the pathogenesis of renal anemia as well as the progression of renal diseases [37, 48]. In particular, Chiang *et al.*[34] have reported that IS, a representative protein-bound uremic toxin, suppresses hypoxia-induced HIF transcriptional activation and subsequent EPO production. In the present study, I investigated the detailed effects and the molecular mechanism of the suppressive effect of IS using HepG2 cells and rats.

I firstly examined whether IS at physiological concentrations in CKD patients (109 ± 61 μM) reported previously [3] affected hypoxia-induced HIF activation and subsequent EPO production *in vitro*. I found that hypoxia- or cobalt chloride-induced expression of *EPO* mRNA was suppressed by IS at 100 μM or higher in EPO-producing HepG2 cells. Moreover, HRE-luciferase reporter gene assay revealed that HIF transcriptional activation induced by hypoxic culture was suppressed by IS at 100 μM or higher, and that induced by cobalt chloride treatment at 20 μM or higher. These results indicate that IS has suppressive effects on hypoxia-induced HIF activation and EPO expression at concentrations similar to the blood levels of CKD patients.

Chiang *et al.*[34] also have reported that oral administration of indole, a metabolic precursor of IS, suppresses EPO production induced by subcutaneous administration of cobalt chloride in rats, indicating that IS has suppressive effects on cobalt

chloride-induced EPO production *in vivo*. Although their report provides *in vivo* evidence of the suppressive effects of IS under a hypoxia mimetic state, it remains unclear whether their results represent the real effect of IS under a physiological hypoxic condition. Therefore, I examined the effect of indole administration on bleeding-induced physiological EPO production in rats. I found that a single oral administration of indole at 100 mg/kg caused the increase in plasma IS concentration up to 140 μM, which was similar to the concentration used in the above-mentioned *in vitro* experiments in HepG2 cells, and the indole administration suppressed the increases in *Epo* mRNA expression in renal cortex tissue and EPO concentration in plasma. These results indicate that IS at concentrations similar to the blood levels of CKD patients suppresses hypoxia-induced EPO production in the kidneys *in vivo*.

Schroeder *et al.*[38] have reported that IS is an endogenous ligand for AHR which is a ligand-dependent transcription factor and belongs to the family of basic helix loop helix (bHLH) and Per-Arnt-Sim (PAS) domain proteins that also includes HIF proteins[49]. Based on this finding, I examined whether the AHR was involved in the suppressive effect of IS on HIF activation in HepG2 cells. I found that inactivation of AHR by either the pharmacological AHR antagonist CH-223191 or AHR-siRNA completely inhibited the suppressive effect of IS on HIF activation. Moreover, IS-induced suppression of *EPO* mRNA expression was inhibited by the AHR antagonist CH-223191. The AHR is a receptor for dioxin-like environmental pollutants[39], therefore, I further examined whether a potent AHR agonist TCDD had similar effect as IS. I found that TCDD strongly suppressed HIF transcriptional activation induced by cobalt chloride treatment and the suppression was completely inhibited by CH-223191. These results demonstrate that the AHR plays a crucial role in the suppressive effect of

IS. Furthermore, nuclear accumulation of HIF-α subunits, an important step for transcriptional activation of HIF [50, 51], was suppressed by IS as well as TCDD, implying that inhibition of nuclear accumulation of HIF-α subunits contributes to IS-induced suppression of HIF activation and subsequent EPO production.

I next investigated the effect of IS on the AHR signaling pathway in both *in vitro* and *in vivo* experiments. Similar to the report by Schroeder *et al.*[38], IS strongly induced *CYP1A1* mRNA expression in HepG2 cells. *CYP1A1* is a representative gene regulated by AHR but the specificity of *CYP1A1* induction in AHR activation is questionable [52]. For this reason, I investigated the translocation of AHR from cytosol to nucleus in HepG2 cells and confirmed that the amount of AHR in the nucleus obviously increased by IS at 20 μM or higher. Moreover, *in vivo* experiments using healthy rats revealed that oral administration of indole to increase blood IS concentration induced the expression of *Cyp1a1* mRNA in both liver and renal cortex tissues and nuclear translocation of AHR in liver tissue. These results demonstrate that IS activates AHR signaling pathway *in vivo* as well as *in vitro*.

As described above, IS suppressed HIF transcriptional activation by inhibiting nuclear accumulation of HIF-α subunits, and IS also induced nuclear translocation of AHR. In the nucleus, both HIF-α subunits and AHR form heterodimeric complexes with ARNT to act as transcription factors. Therefore, I examined the amounts of HIF-α subunits and AHR that heterodimerize with ARNT in the nucleus by co-immunoprecipitation using anti-ARNT antibody. As IS concentration increased, the amount of HIF-α–ARNT complex in the nucleus decreased, while the amount of AHR–ARNT complex increased. In addition, Chilov *et al.*[53] have reported that accumulation of HIF-1α in the nucleus of hypoxic cells was independent of the presence

of ARNT but heterodimerization with ARNT was required for its stable association within the nuclear compartment. These reports and the present results indicate that IS-induced inhibition of nuclear accumulation of HIF-α subunits could be caused by the inhibition of heterodimerization between HIF-α and ARNT. Moreover, Chan *et al.*[54] have reported that AHR competitively inhibits the binding of HIF-1α to ARNT, and *vice versa*. Although HIF-2α is not investigated in this report, HIF-2α is likely to have the same property as HIF-1α based on the structural similarity[55]. Therefore, the present results suggest that in the presence of IS, increased nuclear translocation of AHR competitively inhibits the formation of HIF-α–ARNT complex, which in turn suppresses HIF transcriptional activation and subsequent EPO production.

Taken together, the results of this study demonstrate that IS at clinically relevant concentrations in CKD patients suppresses hypoxia-induced HIF-dependent EPO production both *in vitro* and *in vivo*. Although further studies in CKD model animals and CKD patients are needed, these data suggest that IS contributes to the progression of renal anemia in CKD patients whose blood IS levels increase due to impaired renal excretion. Furthermore, this study is the first to elucidate the detailed molecular mechanism by which AHR plays a crucial role in the suppressive effect of IS. Hence, IS-induced AHR activation may be one of potential therapeutic targets for treating renal anemia.

Tables and Figures

Table 3. *List of primer pairs used in quantitative real-time RT-PCR.*

Gene	Forward (5´-3´)	Reverse (5´-3´)
Human *EPO*	AGCCCAGAAGGAAGCCATCT	GGAAAGTGTCAGCAGTGATTGTTC
Human *CYP1A1*	TCTTCCTTCGTCCCCTTCAC	TGGTTGATCTGCCACTGGTT
Human *HIF1α*	CATAAAGTCTGCAACATGGAAGGT	ATTTGATGGGTGAGGAATGGGTT
Human *HIF2α*	CTGCTCCACGCCCAATAG	GTGCCAGTGTCTCCAAGTC
Human *HPRT*	GACCAGTCAACAGGGGACAT	CGACCTTGACCATCTTTGGA
Rat *Epo*	GCTCCAATCTTTGTGGCATC	ATCCATGTCTTGCCCCCTA
Rat *Cyp1a1*	CCTCTTTGGAGCTGGGTTTG	GCTGTGGGGGATGGTGAA
Rat *Gapdh*	GATGGGTGTGAACCACGAGAAA	ACGGATACATTGGGGGTAGGAA

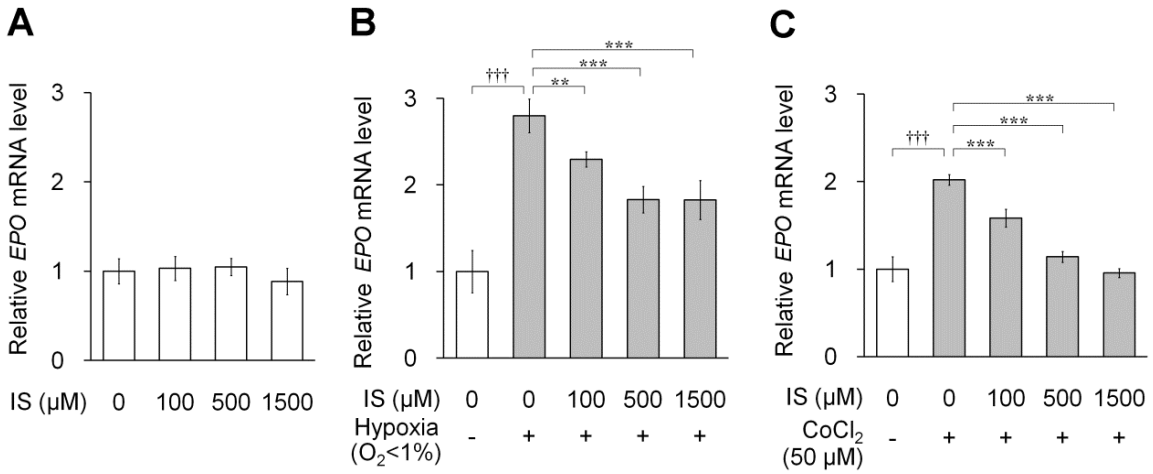

Figure 6. *Effect of IS on hypoxia-induced EPO mRNA expression in HepG2 cells.*

Serum-starved HepG2 cells were treated with IS for 6 hours under normoxic condition (**A**), hypoxic condition (O_2<1%; **B**), or in the presence of 50 μM cobalt chloride ($CoCl_2$; **C**). $CoCl_2$ was added 1 hour after the beginning of IS treatment. After the extraction of total RNA, expression of *EPO* mRNA was measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD of three to four independent experiments. †††, P<0.001 (unpaired t test). **, P<0.01; ***, P<0.001 (Dunnett test). This figure is modified from Asai *et al.*, 2016 [56].

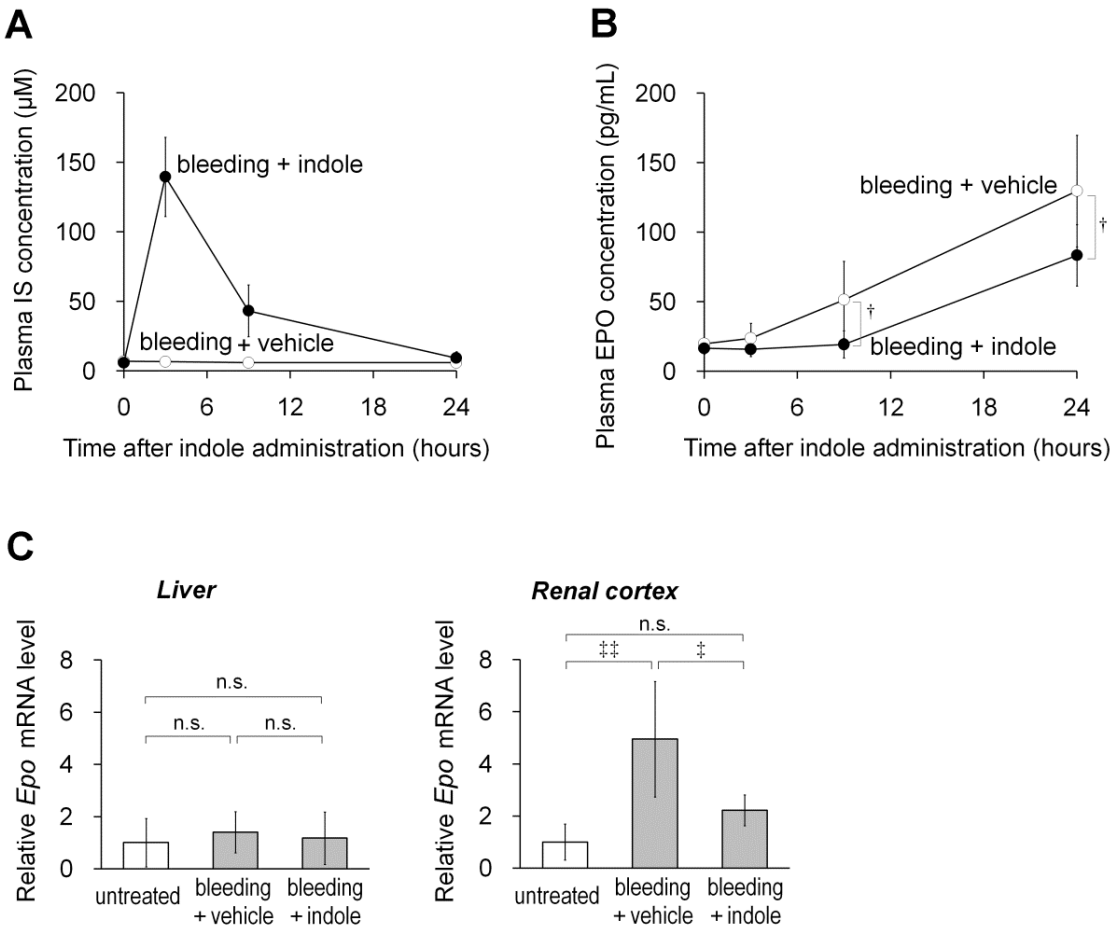

Figure 7. *Effect of single oral administration of indole in the bleeding experiments in rats.*

After blood withdrawal (1 mL/100 g body weight), rats were administrated indole (100 mg/kg) or vehicle (0.5% methylcellulose solution) by oral gavage. (**A**-**B**) Blood sampling was performed 3, 9 or 24 hours after administration, and IS and EPO protein concentrations in plasma were measured by liquid chromatography/electrospray ionization-tandem mass spectrometry and commercial ELISA kit, respectively. Open circle, vehicle control group; closed circle, indole-treated group (n=6 in each group). (**C**) The liver and renal cortex tissues were collected 9 hours after administration. After the extraction of total RNA, *Epo* mRNA expression was measured by quantitative

real-time RT-PCR (n= 6 in each group). Data are expressed as mean ± SD. †, $P<0.05$ (unpaired *t* test).‡, $P<0.05$; ‡‡, $P<0.01$; n.s., not significant (Tukey-Kramer HSD test). This figure is modified from Asai *et al.*, 2016 [56].

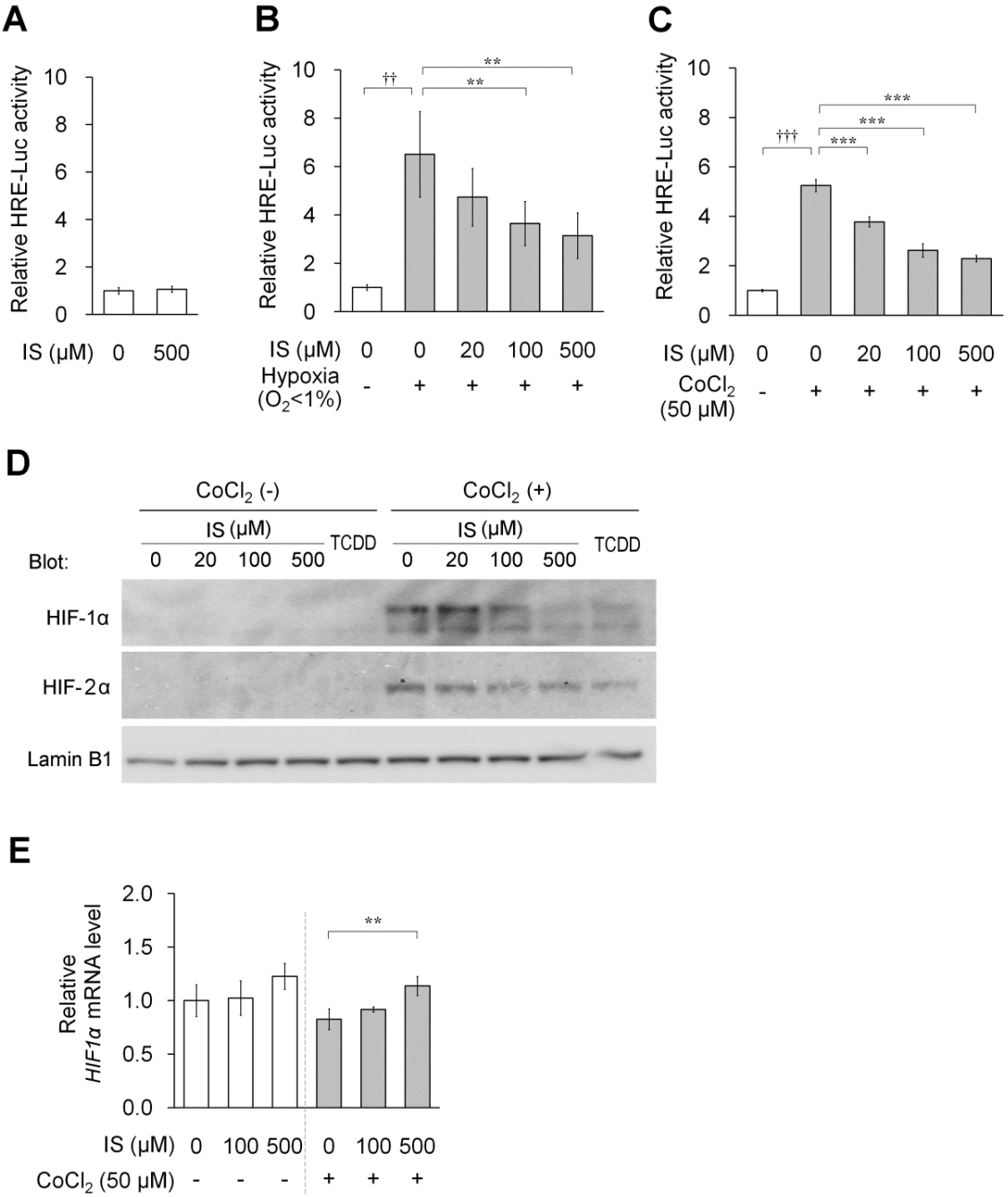

Figure 8. *Effect of IS on HIF transcriptional activation in HepG2 cells.*

(**A**-**C**) HepG2 cells that were transiently transfected with HRE-luciferase reporter gene plasmid together with control plasmid were treated with IS for 5 hours under normoxic condition (**A**), hypoxic condition (**B**), or in the presence of 50 μM $CoCl_2$ (**C**). $CoCl_2$ was

added 1 hour after the beginning of IS treatment. Cellular HRE-luciferase activity was then measured by chemiluminescent assay. Data are expressed as mean ± SD of three to five independent experiments. ††, $P<0.01$; †††, $P<0.001$ (unpaired *t* test). **, $P<0.01$; ***, $P<0.001$ (Dunnett test). (**D**) Cells were treated with IS or TCDD (10 nM) for 4 hours in the presence or absence of 50 μM $CoCl_2$. $CoCl_2$ was added 1 hour after the beginning of IS or TCDD treatment. Nuclear proteins were then extracted and expressions of HIF-1α, HIF-2α and Lamin B1 (loading control) in nuclear protein extracts were detected by immunoblot. Results are representative of three independent experiments with similar results. (**E**) Serum-starved HepG2 cells were treated with IS for 6 hours in the presence or absence of 50 μM $CoCl_2$. $CoCl_2$ was added 1 hour after the beginning of IS treatment. After the extraction of total RNA, expression of *HIF1α* mRNA was measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD of three independent experiments. **, $P<0.01$ (Dunnett test).

This figure is modified from Asai *et al.*, 2016 [56].

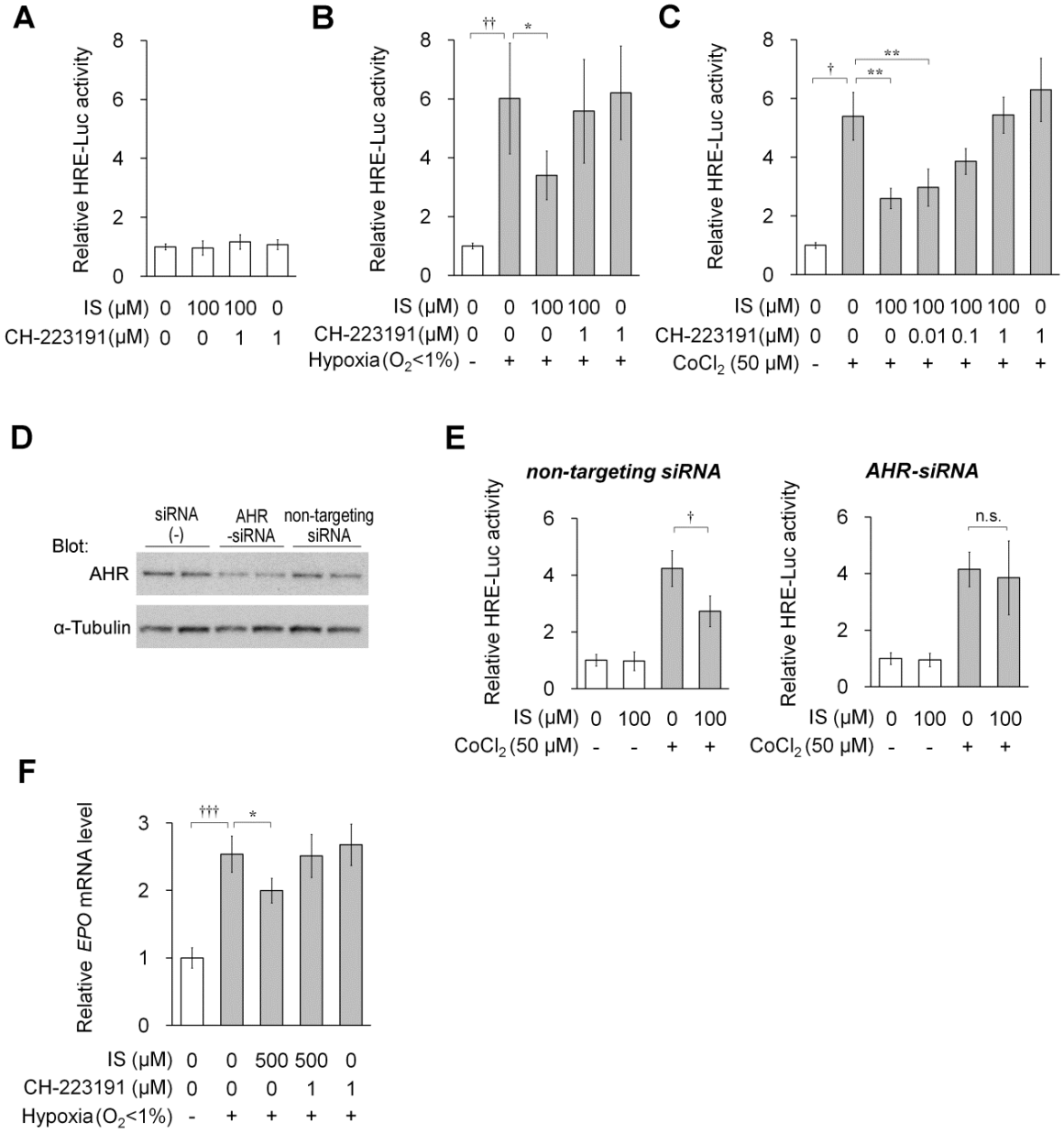

Figure 9. *Effect of AHR inactivation on the IS-induced suppression of HIF transcriptional activation and EPO mRNA expression in HepG2 cells.*

(**A**-**C**) HepG2 cells that were transiently transfected with HRE-luciferase reporter gene plasmid together with control plasmid were treated with IS and/or CH-223191 for 5 hours under normoxic condition (**A**), hypoxic condition (**B**), or in the presence of 50 μM $CoCl_2$ (**C**). $CoCl_2$ was added 1 hour after the beginning of IS treatment. Cellular

HRE-luciferase activity was then measured by chemiluminescent assay. Data are expressed as mean ± SD of three to five independent experiments. †, $P<0.05$; ††, $P<0.01$ (unpaired *t* test). *, $P<0.05$; **, $P<0.01$ (Dunnett test). (**D**) HepG2 cells were transfected simultaneously with HRE-luciferase plasmid and AHR-siRNA or non-targeting siRNA. Cellular expressions of AHR and α-tubulin (loading control) were detected by immunoblot for the evaluation of the knockdown effects of the siRNAs. Results are representative of three independent experiments with similar results. (**E**) The cells transfected with HRE-luciferase plasmid and the siRNAs were treated as described above. Cellular HRE-luciferase activity was measured by chemiluminescent assay. Data are expressed as mean ± SD of three independent experiments. †, $P<0.05$; n.s., not significant (unpaired *t* test). (**F**) Serum-starved HepG2 cells were treated with IS and/or CH-223191 for 6 hours under hypoxic condition ($O_2<1\%$). After the extraction of total RNA, expression of *EPO* mRNA was measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD of five independent experiments. †††, $P<0.001$ (unpaired *t* test). *, $P<0.05$ (Dunnett test).

This figure is modified from Asai *et al.*, 2016 [56].

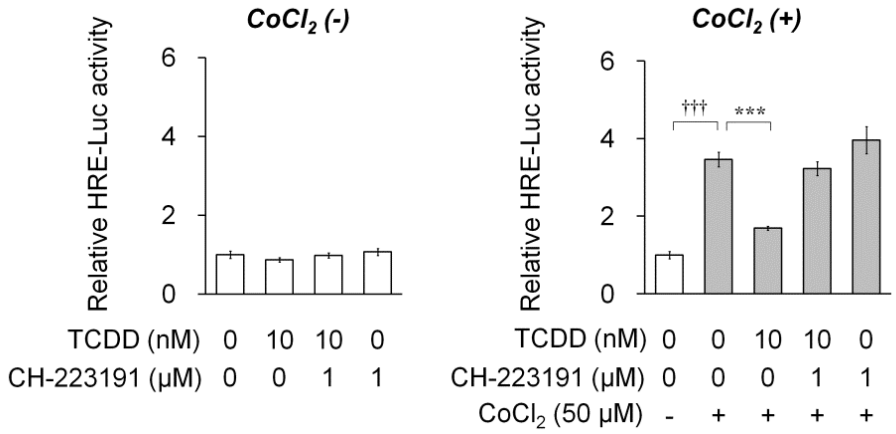

Figure 10. *Effect of TCDD on HIF transcriptional activation in HepG2 cells.*

HepG2 cells that were transiently transfected with HRE-luciferase reporter gene plasmid together with control plasmid were treated with TCDD and/or CH-223191 for 5 hours in the presence or absence of 50 μM $CoCl_2$. $CoCl_2$ was added 1 hour after the beginning of TCDD treatment. Cellular HRE-luciferase activity was measured by chemiluminescent assay. Data are expressed as mean ± SD of three independent experiments. †††, $P<0.001$ (unpaired *t* test); ***, $P<0.001$ (Dunnett test).

This figure is reprinted from Asai *et al.*, 2016 [56].

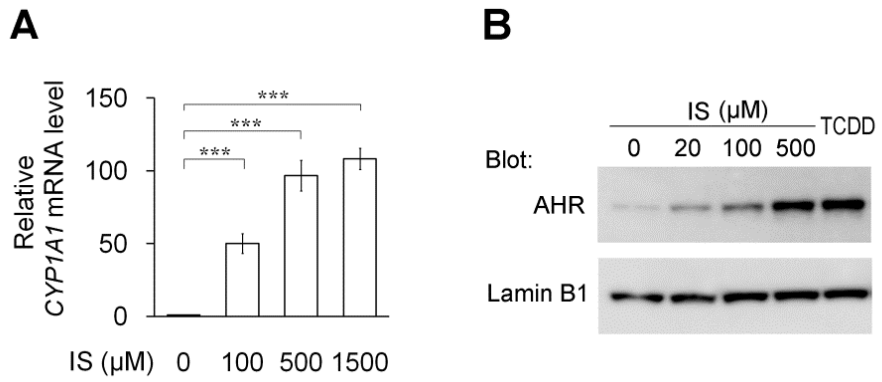

Figure 11. *Effect of IS on the AHR signaling pathway in HepG2 cells.*

(**A**) Serum-starved HepG2 cells were treated with IS for 6 hours. After the extraction of total RNA, expression of *CYP1A1* mRNA was measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD of three independent experiments. ***, $P<0.001$ (Dunnett test). (**B**) HepG2 cells were treated with IS or TCDD (10 nM) for 4 hours, and nuclear proteins were then extracted. Expressions of AHR and Lamin B1 (loading control) in nuclear protein extracts were detected by immunoblot. Results are representative of three independent experiments with similar results.

This figure is modified from Asai *et al.*, 2016 [56].

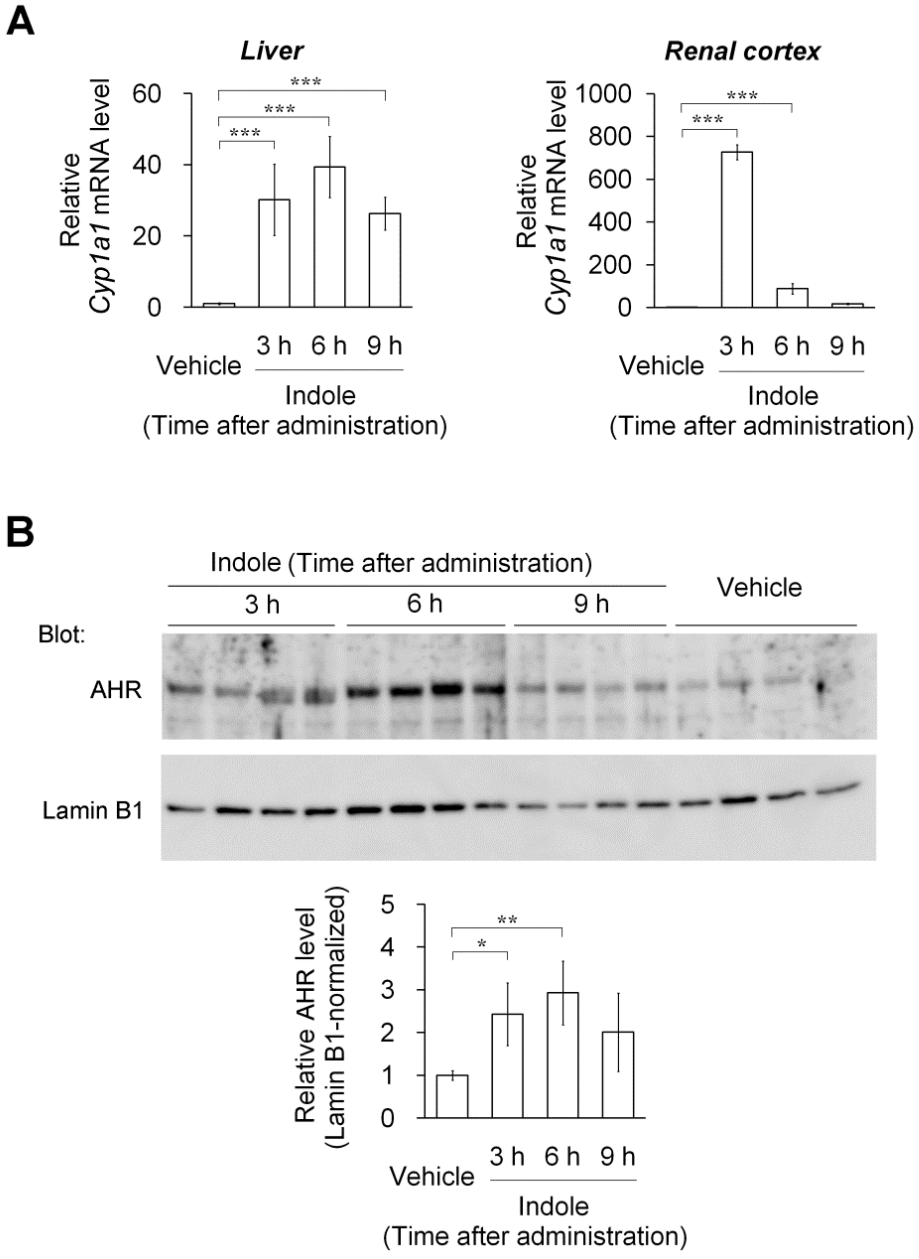

Figure 12. *Effect of single oral administration of indole on the AHR signaling pathway in rats.*

Rats were euthanized 3, 6 or 9 hours after single oral administration of indole (100 mg/kg) and the liver and renal cortex tissues were collected (n=4 in each treatment period). (**A**) After the extraction of total RNA, expression of *Cyp1a1* mRNA in the liver

and renal cortex tissues was measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD. ***, $P<0.001$ (Dunnett test). (**B**) Nuclear proteins were extracted from the liver tissue. Expressions of AHR and Lamin B1 (loading control) in nuclear protein extracts were detected by immunoblot. Semi-quantitative analysis by densitometry was then performed. *, $P<0.05$; **, $P<0.01$ (Dunnett test).

This figure is modified from Asai *et al.*, 2016 [56].

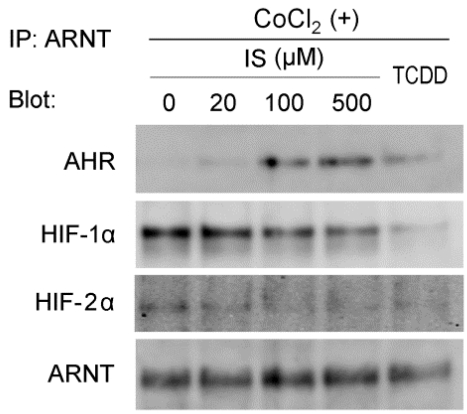

Figure 13. *Effect of IS on the amounts of AHR–ARNT and HIF-α–ARNT complexes in the nucleus of $CoCl_2$-treated HepG2 cells.*

HepG2 cells were treated with IS or TCDD (10 nM) for 4 hours in the presence of 50 μM $CoCl_2$. $CoCl_2$ was added 1 hour after the beginning of IS or TCDD treatment. Nuclear proteins were then extracted and ARNT-bound proteins were immunoprecipitated using anti-ARNT antibody. Expressions of AHR, HIF-1α, HIF-2α and ARNT in the immunoprecipitated samples were detected by immunoblot. Results are representative of three independent experiments with similar results.

This figure is reprinted from Asai *et al.*, 2016 [56].

PART II **Effect of uremic toxins other than indoxyl sulfate on hypoxia-induced erythropoietin expression in HepG2 cells**

Abstract

Patients with chronic kidney disease (CKD) frequently suffer from renal anemia which is mainly caused by inadequate erythropoietin (EPO) production from the kidneys partly due to impairment of intracellular hypoxia-inducible factor (HIF) signaling in renal EPO-producing cells. In Part I, I have shown that indoxyl sulfate (IS), a representative protein-bound uremic toxin increased in the blood of CKD patients, suppresses hypoxia-induced transcriptional activation of HIF and subsequent EPO production through activation of aryl hydrocarbon receptor (AHR). In this part, I further studied the effects of other five protein-bound uremic toxins on HIF-dependent EPO expression using EPO-producing HepG2 cells. I found that indoxyl glucuronide (IG) as well as IS, but not *p*-cresyl sulfate (PCS), phenyl sulfate, 3-indoleacetic acid or hippuric acid, suppressed hypoxia mimetic cobalt chloride-induced expression of *EPO* mRNA. Moreover, IG at clinically relevant concentrations in CKD patients suppressed the HIF transcriptional activation induced by both cobalt chloride treatment and hypoxic culture. IG also induced *CYP1A1* mRNA expression and nuclear translocation of AHR protein, indicating that IG activates AHR signaling pathway. Blockade of AHR by a pharmacological antagonist CH-223191 inhibited the IG-induced suppression of HIF transcriptional activation. Taken together, this study is the first to elucidate the biological effects of IG to suppress HIF-dependent EPO production through activation of AHR. These data suggest that not only IS but also IG contributes to the impairment of HIF signaling in renal anemia.

Introduction

The results of the previous study described in Part I indicate that IS-induced AHR activation is one of the molecular mechanisms causing the impairment of intracellular HIF signaling in EPO-producing cells. On the other hand, apart from IS, there are many kinds of compounds considered as protein-bound uremic toxins that could contribute to the progression of CKD. As similar to IS, PCS, a metabolite of tyrosine, has been reported to be involved in the progression of CKD and cardiovascular diseases associated with CKD through inducing oxidative stress [18-20]. Furthermore, a clinical study suggests that PCS may be involved in renal anemia [57]. In addition, 3-indoleacetic acid and hippuric acid have also been reported to exacerbate the progression of CKD in animal experiments [21]. These compounds may also have a role in the impairment of intracellular HIF signaling in EPO-producing cells in renal anemia. Furthermore, it is possible that other protein-bound uremic toxins of which biological effects are unknown contribute to the progression of renal anemia; however, none of the studies have focused on their effects on HIF signaling and EPO production.

In this study, I evaluated five protein-bound uremic toxins including IG, PCS, phenyl sulfate, 3-indoleacetic acid and hippuric acid, regarding their potential to suppress the HIF-dependent EPO expression using the human hepatoma cell line HepG2 which retains the ability to express EPO in response to hypoxia. Furthermore, I investigated the involvement of AHR in the suppression of HIF activation for the elucidation of the mechanism of action.

Materials and Methods

1. Uremic toxins

The chemical structures of protein-bound uremic toxins investigated in this study are shown in Figure 14. IS potassium salt and IG cyclohexylammonium were purchased from Alfa Aesar (Ward Hill, MA, USA) and Glycosynth (Warrington, Cheshire, UK), respectively. 3-Indoleacetic acid potassium salt and hippuric acid sodium salt were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PCS sodium salt and phenyl sulfate sodium salt (>98% purity) were synthesized at Eiweiss (Shizuoka, Japan). All these uremic toxins were dissolved in PBS, passed through a sterile filter (Millex-GP, 0.22 μm; Millipore, Burlington, MA, USA) and then diluted with culture medium.

2. Cell culture

The human hepatoma cell line HepG2 was obtained from DS Pharma Biomedical (Osaka, Japan). The cells were cultured in MEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT, USA), 1% non-essential amino acid solution (Sigma-Aldrich), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Tokyo, Japan) at 37°C in a 5% CO_2 humidified incubator unless otherwise indicated. For induction of cellular hypoxic response, HepG2 cells were treated with cobalt chloride (Wako Pure Chemical Industries, Osaka, Japan) at 50 μM. Alternatively, cells were cultured under hypoxic condition using an AnaeroPack System (Mitsubishi Gas Chemical, Tokyo, Japan) for periods indicated in the figure legends. Uremic toxins were added 1 hour prior to cobalt

chloride treatment or immediately before hypoxic culture.

3. Cell viability assay

HepG2 cells (1×10^4/well) were seeded into 96-well plates and cultured overnight. Cells were then treated with uremic toxins (0, 500, 1500 or 5000 μM) for 24 hours in the presence or absence of cobalt chloride. MTT (Dojindo Laboratories, Kumamoto, Japan) was added at the end of treatment with uremic toxins, and cells were further cultured for 1 hour. The formazan crystals were dissolved in DMSO, and absorbance at 570 nm was determined using a microplate reader.

4. RNA isolation and quantitative real-time RT-PCR

HepG2 cells (2.5×10^5/well) were seeded into 12-well plates and cultured overnight. The culture medium was replaced with MEM containing 0.1% FBS, and cells were further cultured overnight under serum starvation. Serum-starved cells were then treated with uremic toxins, and cultured with or without cobalt chloride or under hypoxic condition. Total RNA was extracted using an Illustra RNA Spin Mini Kit (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. Total RNA (250 ng in 10 μL reaction volume) was reverse transcribed with random hexamer primers using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). Quantitative real-time RT-PCR was then performed with fivefold-diluted cDNA using a KAPA SYBR fast qPCR kit (Nippon Genetics, Tokyo, Japan) and gene-specific primer pairs (35-40 cycles at 95ºC for 3 s, 60ºC for 30 s). The sequences of primers used are shown in Table 3 in Part I. Values for the mRNA of *EPO*, *CYP1A1*, *HIF1α* and *HIF2α* were normalized to the expression of *HPRT* mRNA. Analyses were conducted with a

Thermal Cycler Dice Real Time System (Takara Bio).

5. Measurement of HIF transcriptional activity by luciferase reporter gene assay

The luciferase reporter plasmid for HRE (pGL4.42 [*luc2P*/HRE/Hygro] Vector) and control plasmid (pGL4.74 [*hRluc*/TK] Vector) were obtained from Promega (Madison, WI, USA). After seeding into 96-well plate (1×10^4/well) and overnight culture, cells were co-transfected with both the HRE-luciferase and control plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche diagnostics, Tokyo, Japan) and cultured for 24 hours. Cells were then treated with uremic toxins and cultured with or without cobalt chloride or under hypoxic condition for periods indicated in the figure legends. Cells were washed with ice-cold PBS, and cellular luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega) with a Lumat LB9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). HIF transcriptional activity is expressed as ratio of *Photinus pyralis* luciferase activity / *Renillareniformis* luciferase activity. In some experiments, the AHR antagonist CH-223191 (Sigma-Aldrich) dissolved in DMSO was added at the same time of treatment with uremic toxins. The final concentration of DMSO in the medium was set to less than 0.1% in all experiments.

6. Nuclear protein extraction and immunoblot analysis

HepG2 cells (7.5×10^6/dish) were seeded into 10-cm dishes and cultured overnight. After treatment with uremic toxins and cobalt chloride for 4 hours, cells were harvested. Nuclear protein extraction was then performed using NE-PER Nuclear and

Cytoplasmic Extraction Reagents supplemented with Halt Protease Inhibitor Cocktail with EDTA (both from Thermo Fisher Scientific, Kanagawa, Japan) according to the manufacturer's instructions. Protein concentrations of the nuclear protein extracts were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific) with bovine serum albumin as standard. Equal amounts (5 μg) of nuclear protein extracts were mixed with reducing SDS sample buffer and heated for 3 min at 100°C. The samples were separated by SDS-PAGE and transferred to a PVDF membrane (GE Healthcare). After blocking with 3% skim milk solution, the membranes were treated with one of the following primary antibodies: anti-AHR mouse monoclonal antibody (A-3; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-Lamin B1 rabbit polyclonal antibody (Medical & Biological Laboratories, Aichi, Japan). After treatment with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), signals were visualized using an ECL prime detection kit (GE Healthcare) and a luminescence imager Light-Capture AE-6981 (Atto, Tokyo, Japan).

7. Statistical analysis

Two-tailed unpaired *t* test was conducted for two-sample comparison using Excel 2010 (Microsoft, Tokyo, Japan). For multiple-sample comparisons, Dunnett test was conducted using JMP (ver. 10.0.0, SAS Institute, Cary, NC, USA). Differences were considered statistically significant for *P* values less than 0.05.

Results

1. IG but not PCS, phenyl sulfate, 3-indoleacetic acid or hippuric acid suppresses cobalt chloride-induced increase of *EPO* mRNA expression

IG, PCS, phenyl sulfate, and 3-indoleacetic acid had no influence on the viability of HepG2 cells at concentrations up to 1500 μM, and hippuric acid had no cell toxicity up to 5000 μM which was the highest concentration investigated, both in the presence and absence of cobalt chloride (data not shown). Therefore, the highest concentration of these uremic toxins was set at 1500 μM in subsequent experiments to evaluate the potential to suppress *EPO* mRNA expression. IS was also used as a positive control at 500 μM, the concentration at which definitive effects were observed in experiments described in Part I.

In the absence of hypoxic stimulation, IG, PCS, phenyl sulfate, 3-indoleacetic acid and hippuric acid at up to 1500 μM as well as IS at 500 μM had no effect on the expression of *EPO* mRNA (Figure 15). Under hypoxia mimetic condition induced by cobalt chloride treatment, *EPO* mRNA expression increased by approximately 2-fold. The hypoxia-induced increase in *EPO* mRNA expression was significantly suppressed by IG in a concentration-dependent manner. IS also significantly suppressed the cobalt chloride-induced *EPO* mRNA expression. On the other hand, the other four uremic toxins had no significant effect on *EPO* mRNA expression under hypoxia mimetic condition.

In experiments using lower concentrations of IG, IG significantly suppressed cobalt chloride-induced increase in *EPO* mRNA expression at 100 μM or higher in a concentration-dependent manner (Figure 16A). Under hypoxic culture conditions

(O_2<1%), *EPO* mRNA expression increased by 2.8-fold and this increase was significantly suppressed by IG at 100 μM or higher (Figure 16B). On the other hand, mRNA expressions of HIF-α subunits (*HIF1α* and *HIF2α*) were not affected by IG at up to 500 μM in the presence of cobalt chloride (Figure 16C and 16D).

2. IG suppresses hypoxia-induced HIF transcriptional activation

Transcriptional activity of HIF was assayed by measuring HRE-luciferase activity. In the absence of hypoxic stimulation, IG had no effect on HRE-luciferase activity (Figure 17A). Hypoxic culture (O_2<1%) caused an increase in HIF transcriptional activity by 2.2-fold and this increase was significantly suppressed by IG at 30 μM or higher (Figure 17B). Likewise, HIF transcriptional activity increased to 3.9-fold in cobalt chloride-induced hypoxia mimetic condition, and this increase was significantly suppressed by IG even at 10 μM, the lowest concentration investigated (Figure 17C). In addition, IS also significantly suppressed HIF activation induced by hypoxic culture and cobalt chloride treatment (Figure 17B and 17C).

3. IG activates AHR signaling pathway

IG strongly induced the expression of *CYP1A1* mRNA, one of the major genes regulated by AHR, by 70-fold at 100 μM (Figure 18A). Moreover, immunoblot analysis revealed that IG as well as IS increased the amount of AHR in the nuclear protein extracts (Figure 18B), indicating that IG activates the AHR signaling pathway.

4. AHR antagonist inhibits the suppressive effect of IG on HIF activation

As shown in Figure 19, the increase in HIF transcriptional activity induced by

hypoxic culture (O_2<1%) was significantly suppressed by IG (100 μM), and IG-induced suppression was completely inhibited by co-treatment with an AHR antagonist CH-223191 (1 and 10 μM), while CH-223191 alone had no influence on hypoxia-induced HIF activation. Likewise, the increase in HIF transcriptional activity induced by cobalt chloride treatment was significantly suppressed by IG (100 μM), and this IG-induced suppression was completely inhibited by co-treatment with CH-223191.

Discussion

Impaired HIF signaling in renal EPO-producing cells is one of causes of inadequate EPO production in renal anemia. Previously I, as described in Part I, and others [34] have reported that IS, a representative protein-bound uremic toxin that accumulates in the blood of CKD patients, suppresses hypoxia-induced HIF transcriptional activation and subsequent EPO production in HepG2 cells and rats, suggesting that IS plays a role in the impaired HIF signaling in renal anemia. In the present study, using HepG2 cells, I evaluated other protein-bound uremic toxins: IG, PCS, phenyl sulfate, 3-indoleacetic acid and hippuric acid, regarding their potential to suppress hypoxia-induced HIF activation and EPO expression.

I firstly examined the effect of these uremic toxins at concentrations up to 1500 μM, which was the highest concentration without causing cell toxicity, on cobalt chloride-induced *EPO* mRNA expression to determine their potential to inhibit the HIF signaling pathway. I found that only IG (and positive control IS) suppressed the increase in *EPO* mRNA expression under hypoxia mimetic condition at 500 μM or higher, while the other four uremic toxins had no effect up to 1500 μM. Itoh *et al.*[58] have reported that concentrations of these uremic toxins in the blood of hemodialysis patients (ESRD patients) range from 10 to 200 μM. Based on these findings, the uremic toxins other than IG and IS are considered to have less or no potential to suppress the HIF signaling pathway in physiological conditions.

Since blood levels of IG in hemodialysis patients have been reported to be 11.7 ± 8.5 μM [58], I next investigated the effect of IG at lower concentrations including the physiological range. The hypoxia- or hypoxia mimetic cobalt chloride-induced

increase in *EPO* mRNA expression was suppressed by IG at 100 μM or higher. Moreover, HRE-luciferase reporter assay revealed that IG suppressed HIF transcriptional activation induced by cobalt chloride treatment at 10 μM or higher, and at 30 μM or higher suppressed HIF activation induced by hypoxic culture. These results indicate that IG suppresses hypoxia-induced HIF transcriptional activation at a concentration range found in the blood of CKD patients.

To investigate the possibility that suppression of HIF transcriptional activation by IG is due to inhibition of endogenous expression of HIF-1α and HIF-2α, I examined the effect of IG on mRNA expression of these genes. In the presence of cobalt chloride, IG had no effect on mRNA expressions of both *HIF-1α* and *HIF-2α* even at 500 μM, the highest concentration examined. Consequently, the suppression by IG is due to mechanisms other than inhibition of endogenous expression of HIF-α subunits.

As described in Part I, IS activates AHR both *in vitro* and *in vivo*, and the ligand activity of IS with AHR is indispensable for the suppressive effects of IS on hypoxia-induced HIF transcriptional activation and *EPO* mRNA expression. This previous study also suggests that IS-induced increase in AHR protein in the nuclear compartment competitively inhibits the formation of HIF-α–ARNT complex. Based on these findings, I further examined the contribution of AHR to the suppressive effects of IG. I found that IG strongly induced the expression of *CYP1A1* mRNA and nuclear translocation of AHR protein, indicating that IG activates the AHR signaling pathway. Furthermore, blockade of AHR by the pharmacological antagonist CH-223191 inhibited the suppressive effect of IG on transcriptional activation of HIF. These results indicate that similar to IS, the IG-induced activation of AHR signaling pathway plays a crucial role in the suppressive effect of IG on hypoxia-induced HIF activation.

Several reports have shown that 3-indoleacetic acid induces pro-inflammatory response and procoagulant tissue factor production in human endothelial cells through activating AHR [59, 60]. Because of this, 3-indoleacetic acid is also recognized as an AHR ligand like IS [61, 62]. However, contrary to my findings with IG and IS, 3-indoleacetic acid had no effects on hypoxia-induced *EPO* mRNA expression in this study, suggesting that 3-indoleacetic acid has less or no effects on AHR. The difference in these findings could be related to the cell type used and/or cellular uptake of this compound, and further studies using other EPO-producing cells are needed to confirm the effects of 3-indoleacetic acid on HIF-dependent EPO expression. With regard to IG, it has also been described as an AHR ligand in review articles [61, 62]; however, no source data and papers exist to the best of my knowledge. Therefore, I believe that the present study is the first to make clear the AHR-activating effects of IG.

Taken together, the results of this study demonstrate that IG at clinically relevant concentrations in CKD patients suppresses hypoxia-induced transcriptional activation of HIF through activation of AHR signaling pathway. Although IG has been known to be elevated in the blood of CKD patients for the past two decades, its biological effects remain obscure for a long time [63]. The present study is the first to elucidate that IG may be involved in impaired HIF signaling in renal anemia, which is an important complication associated with CKD. Although further studies in CKD model animals and patients are required, my data suggest that not only IS but also IG contributes to the pathogenesis of renal anemia in CKD patients.

Figures

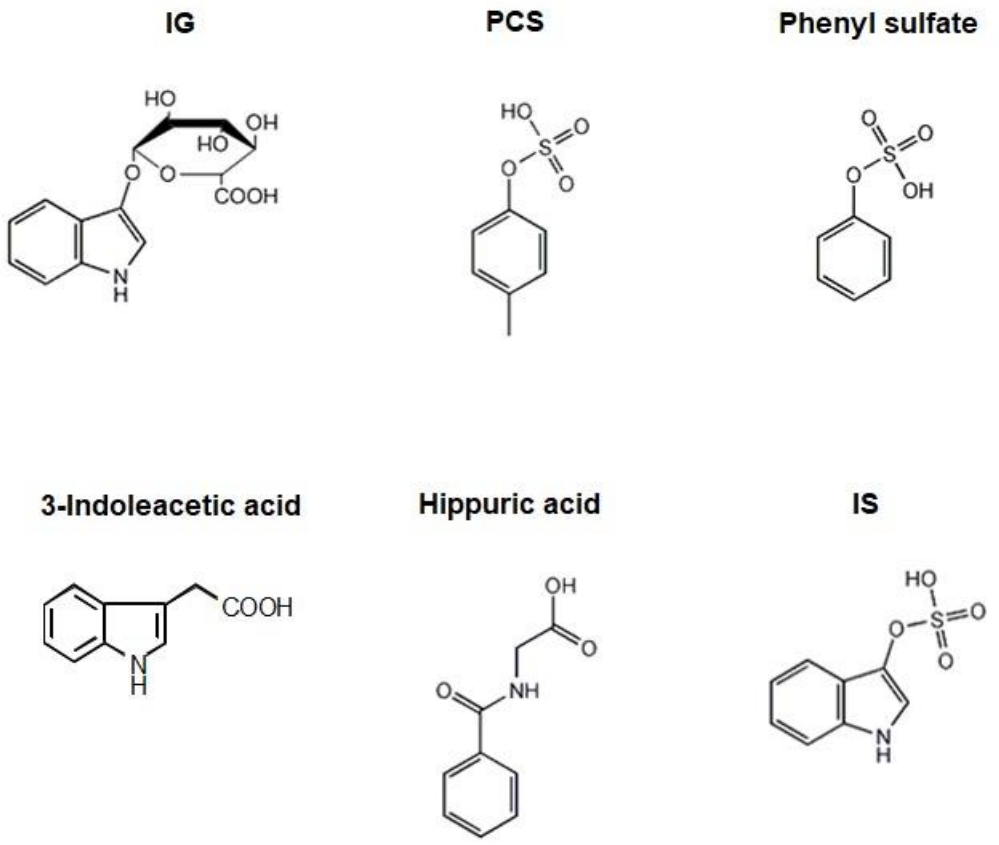

Figure 14. *Chemical structures of protein-bound uremic toxins investigated in this study.*

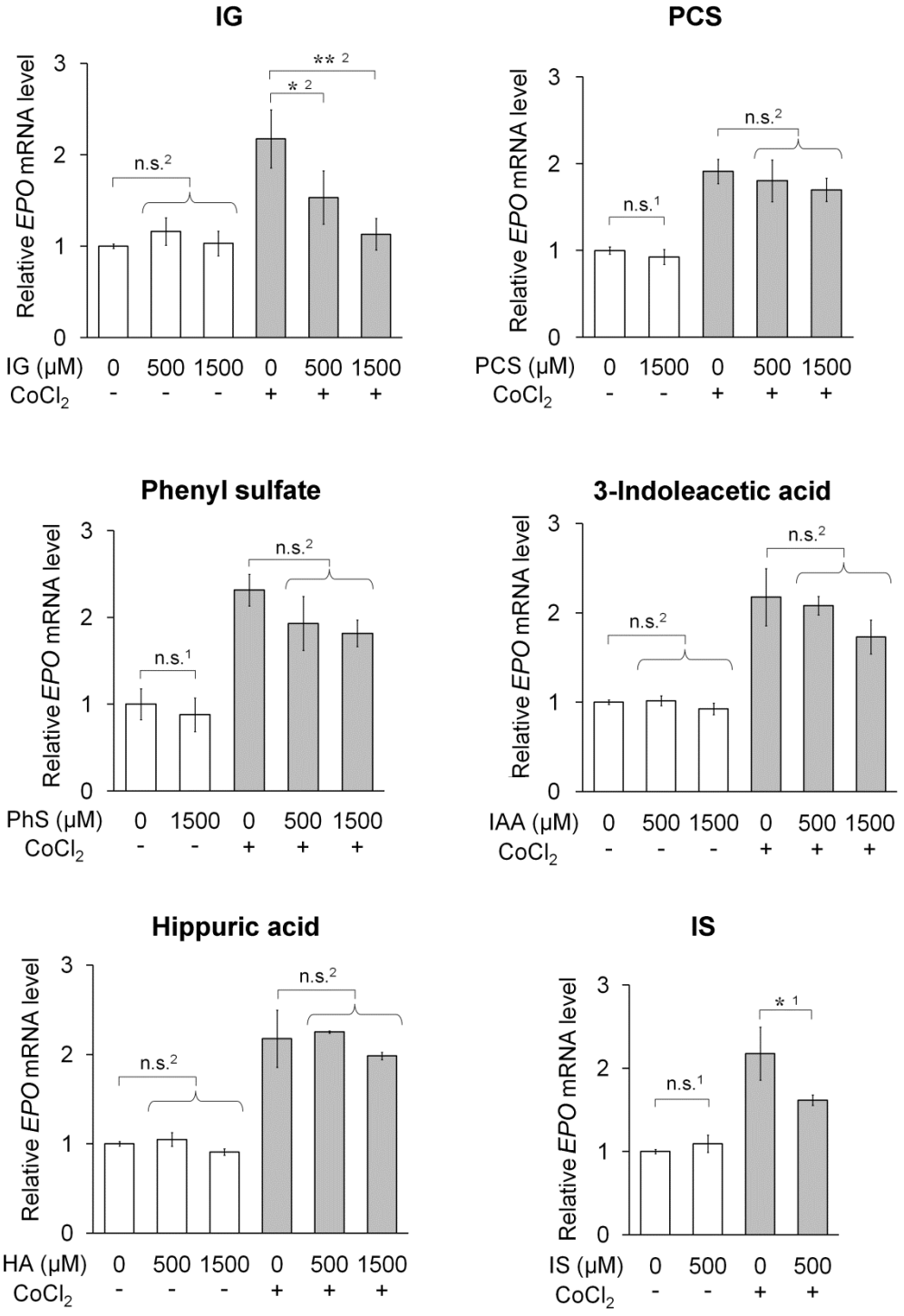

Figure 15. *Effects of protein-bound uremic toxins on hypoxia-induced EPO mRNA expression.*

Serum-starved HepG2 cells were treated with IG, PCS, phenyl sulfate (PhS),

3-indoleacetic acid (IAA), hippuric acid (HA) or IS for 6 hours in the presence or absence of cobalt chloride ($CoCl_2$; 50 μM). $CoCl_2$ was added 1 hour after the beginning of treatment with uremic toxins. After the extraction of total RNA, expression of *EPO* mRNA was measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant ([1], unpaired *t* test; [2], Dunnett test).

This figure is modified from Asai *et al.*, 2018 [64].

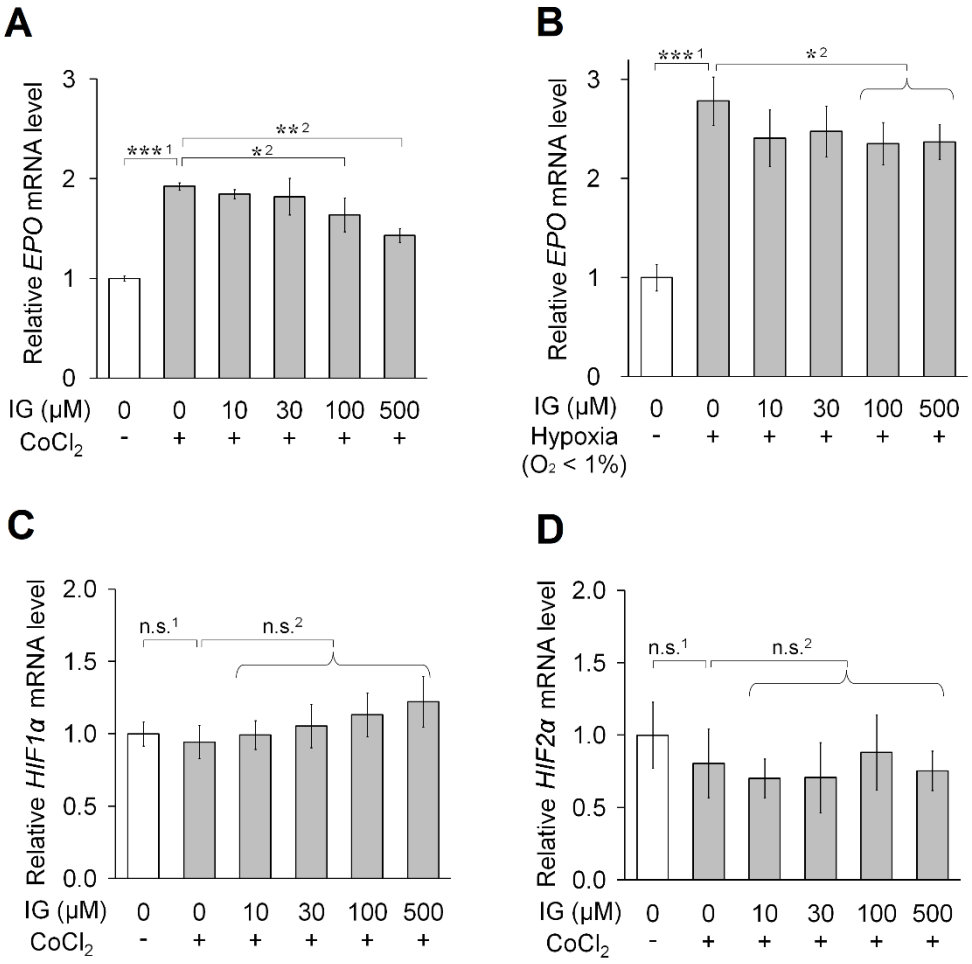

Figure 16. *Effects of IG at lower concentrations on mRNA expressions of EPO, HIF1α and HIF2-α under hypoxic stimulation.*

Serum-starved HepG2 cells were treated with IG for 6 hours in the presence of 50 μM $CoCl_2$ (**A**, **C**, **D**) or under hypoxic condition (O_2<1%; **B**). $CoCl_2$ was added 1 hour after the beginning of IG treatment. After the extraction of total RNA, the mRNA expressions of *EPO* (**A**, **B**), *HIF1α* (**C**) and *HIF2α* (**D**) were measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD of three to five independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ([1], unpaired *t* test; [2], Dunnett test).

This figure is modified from Asai *et al.*, 2018 [64].

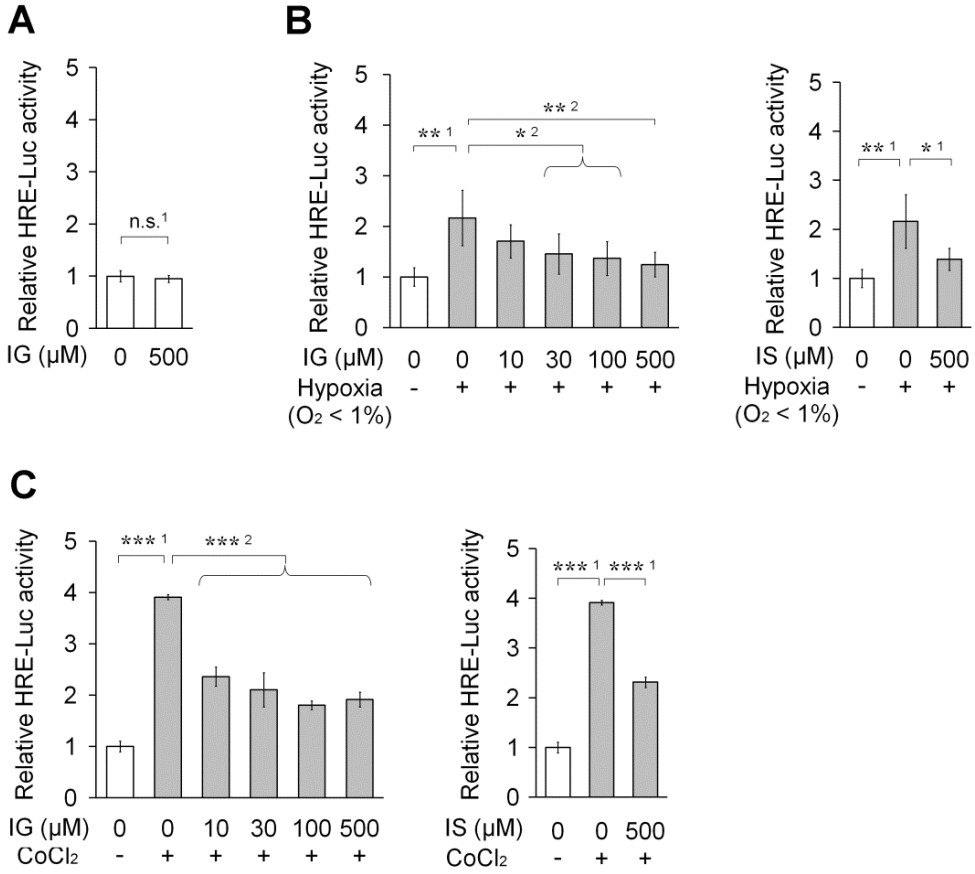

Figure 17. *Effects of IG on hypoxia-induced HIF transcriptional activation.*

HepG2 cells that were transiently transfected with HRE-luciferase reporter gene plasmid together with control plasmid were treated with IG or IS for 5 hours under normoxic condition (**A**), hypoxic condition (O_2<1%; **B**), or in the presence of 50 μM $CoCl_2$ (**C**). $CoCl_2$ was added 1 hour after the beginning of IG or IS treatment. Cellular HRE-luciferase activity was measured by chemiluminescent assay. Data are expressed as mean ± SD of three to five independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ([1], unpaired *t* test; [2], Dunnett test).

This figure is reprinted from Asai *et al.*, 2018 [64].

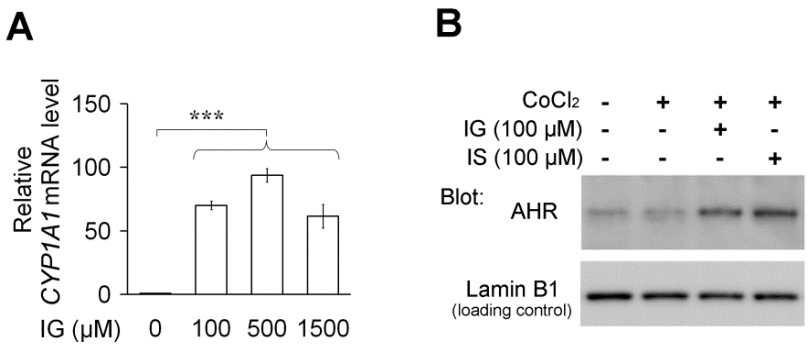

Figure 18. *Effect of IG on the AHR signaling pathway.*

(**A**) Serum-starved HepG2 cells were treated with IG for 6 hours. After the extraction of total RNA, expression of *CYP1A1* mRNA was measured by quantitative real-time RT-PCR. Data are expressed as mean ± SD of three independent experiments. ***, $P < 0.001$ (Dunnett test). (**B**) HepG2 cells were treated with IG or IS for 4 hours in the presence of 50 μM $CoCl_2$. $CoCl_2$ was added 1 hour after the beginning of IG or IS treatment. Nuclear proteins were then extracted and expressions of AHR and Lamin B1 (loading control) proteins in the nuclear protein extracts were detected by immunoblot. Results are representative of three independent experiments with similar results.

This figure is modified from Asai *et al.*, 2018 [64].

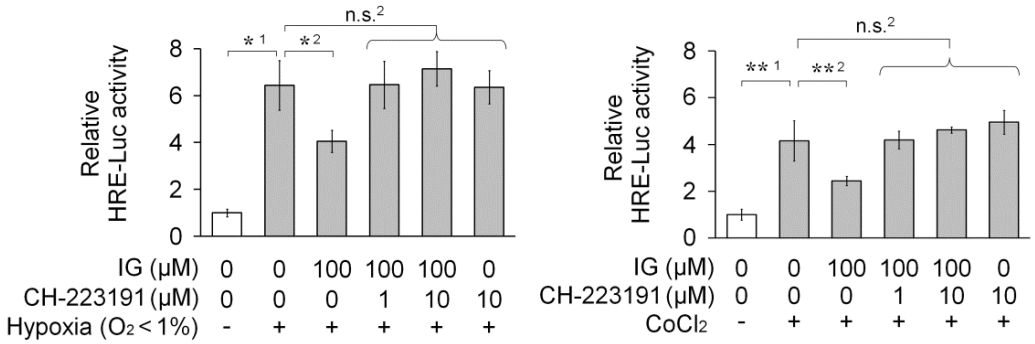

Figure 19. *Effect of AHR antagonist CH-223191 on IG-induced suppression of HIF transcriptional activation.*

HepG2 cells that were transiently transfected with HRE-luciferase reporter gene plasmid together with control plasmid were treated with IG and/or the AHR antagonist CH-223191 for 5 hours under hypoxic culture condition (O_2<1%; left panel) or in the presence of 50 μM $CoCl_2$ (right panel). $CoCl_2$ was added 1 hour after the beginning of IG treatment. Cellular HRE-luciferase activity was then measured by chemiluminescent assay. Data are expressed as mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant ([1], unpaired *t* test; [2], Dunnett test).

This figure is modified from Asai *et al.*, 2018 [64].

General Discussion

Renal dysfunction leads to accumulation of uremic toxins in CKD patients. In particular, protein-bound uremic toxins are believed to be involved in the progression of CKD and CKD-associated complications such as cardiovascular disease, bone disorder, and renal anemia based on their property that less efficiently removed by hemodialysis. Various studies have been conducted for many years to elucidate their biological effects on the renal function [10-12, 19, 21], cardiovascular cells [13-16, 18, 20], bone metabolism [65, 66], and renal anemia [34]. However, most of these researches have paid attention to only a few representative protein-bound uremic toxins such as IS and PCS, and compounds of which biological effects have been elucidated are limited. Furthermore, even in IS and PCS, the mechanism of action of their biological effects is unknown other than the induction of oxidative stress. In this study, I have focused on the impairment of EPO production which is the principal cause of renal anemia, and studied the effects and mechanisms of IS and other protein-bound uremic toxins to clarify their involvement in the pathogenesis of renal anemia.

The results of the present study described in Part I and II revealed that two protein-bound uremic toxins IS and IG suppressed hypoxia-induced transcriptional activation of HIF and subsequent *EPO* mRNA expression at physiological concentrations in CKD patients. On the contrary, PCS, phenyl sulfate, 3-indoleacetic acid or hippuric acid had no suppressive effects on hypoxia-induced *EPO* mRNA expression even at higher concentrations than physiological level, indicating that these compounds had less or no potential to suppress the HIF signaling pathway. Mechanistic experiments to determine the molecules/pathways that contribute to the suppressive effects of IS and IG revealed that both compounds have agonistic effect on AHR similar to a representative AHR agonist TCDD and blockage of AHR by pharmacological

antagonist and/or siRNA for AHR resulted in abolishment of their suppressive effect on HIF activation. These results demonstrate that activation of AHR signaling pathway induced by IS and IG plays an indispensable role in the suppressive effect on hypoxia-induced HIF activation.

The mechanisms for the suppression of HIF activation by AHR are considered as follows. HIF transcription factor is a heterodimeric complex composed of HIF-α subunits (HIF-1α and HIF-2α) and ARNT. HIF-α subunits normally exist in the cytoplasm, and under normoxic condition it is rapidly hydroxylated by PHD and degraded by the ubiquitin-proteasome pathway. Under hypoxic condition, HIF-α escapes from the degradation pathway, which in turn accelerates its nuclear accumulation and dimerization with ARNT to stable association within the nuclear compartment [53]. Then, the heterodimeric complex HIF binds to HRE and promotes the expression of target genes including *EPO* [28]. On the other hand, AHR exists in the cytoplasm as an inactive form. Upon binding ligands including IS and IG, AHR translocates into the nucleus, dimerizes with ARNT to induce the expression of target genes as similar to HIF-α [39]. Based on this basic knowledge about intracellular dynamics of these molecules, it is considered that increased AHR in nucleus induced by the ligands IS and IG competitively inhibits the binding of HIF-α to ARNT, which in turn suppresses HIF transcriptional activation and subsequent EPO expression (Figure 20). Furthermore, this interpretation is supported by the results of electrophoretic mobility shift assay reported by Chan *et al.* [54], showing that addition of AHR protein into the mixture of HIF-1α and ARNT proteins and the oligonucleotide containing the target sequence for HIF inhibits the formation of HIF-1α–ARNT complex and binding to the target sequences.

Clinically, erythropoiesis stimulating agents (ESAs) which are recombinant EPO proteins or its derivatives are administrated for the treatment of renal anemia to replace the inadequate endogenous production. However, there are several disadvantages with this therapy such as only injections and high medical expenses. Especially, the most important point is the increased risks of cardiovascular disease by excessive amount of EPO [67]. For this reason, there are strong demands of new therapeutic approaches for the prevention and treatment of renal anemia. The studies described in this thesis have elucidated that IS- and IG-induced AHR activation impairs HIF activation and subsequent EPO production. These results suggest that protein-bound uremic toxins-induced AHR activation may be one of potential therapeutic targets for treating renal anemia. Accordingly, further investigations in CKD model animals and patients should be performed to identify the therapeutic effects of the inhibition of AHR. In addition, Dou *et al.* [68] have reported in 2018 that AHR is activated in whole blood from patients with CKD and in the aorta and heart of CKD model mice, indicating that AHR is activated systemically. Therefore, protein-bound uremic toxins-induced AHR activation may contribute not only to renal anemia but also to other CKD-associated complications such as cardiovascular disease and mineral and bone disorders.

In recent years, low molecular weight inhibitors of PHD, a key enzyme for HIF inactivation, are under intense investigation as a new therapeutic drug for renal anemia. PHD inhibitors inhibit the intracellular HIF degradation pathway and forcibly activate HIF signaling to produce endogenous EPO. The first PHD inhibitor roxadustat has been launched in China in 2018 and more recently has also been approved in Japan in 2019. Clinical trials demonstrate that roxadustat has improvement effects on renal anemia in

patients with CKD equivalent to ESAs [69, 70]. Furthermore, this compound is superior to ESAs in terms to be orally available, and thus probably takes a leading part in the treatment of renal anemia. On the other hand, since the mode of action of PHD inhibitors utilizes intracellular HIF signaling pathway in the nucleus, it is assumed that the HIF activation and EPO production by PHD inhibitors is still affected by AHR. In other words, the protein-bound uremic toxins-mediated AHR activation may be an obstacle for the treatment with PHD inhibitors. Therefore, future studies should investigate the relation between the therapeutic response to PHD inhibitors and the amounts of the uremic toxins in the body of CKD patients.

Production of erythrocytes and destruction of degenerated/aged erythrocytes constantly occur in the body and anemia develops as the result of the abnormalities of the balance of the homeostasis. Whereas decreased erythropoiesis in the bone marrow due to the inadequate EPO production from the kidneys is the most important mechanism in the pathogenesis of renal anemia, other mechanisms in erythrocyte homeostasis also contribute to the onset and progression of renal anemia.

With regard to erythropoiesis in the bone marrow, mouse bone marrow cell culture experiments revealed that some protein-bound uremic toxins (putrescine, spermidine, and spermine; collectively called polyamines) inhibited EPO-induced differentiation of erythrocyte progenitors [36]. In addition, Adelibieke *et al.* [71] have reported that IS attenuates EPO-induced tyrosine phosphorylation of EPO receptor and the downstream Akt signaling in human umbilical vein endothelial cells (HUVECs). These reports suggest that protein-bound uremic toxins have suppressive effects not only on the process of EPO production but also on the physiological reaction to EPO.

As for the process of the degeneration and destruction of erythrocytes, the erythrocyte life span of CKD patients under dialysis has been reported to be shortened [35, 72], and enhanced extravascular hemolysis due to the increase of phosphatidylserine-positive erythrocytes which are recognized and phagocytized by macrophages is proposed for a possible cause [73]. These reports suggest that some causes related to CKD facilitate the degeneration and destruction of peripheral erythrocytes. Regarding the effects of protein-bound uremic toxins on the degeneration and destruction process, Ahmed *et al.* [74, 75] have reported that IS and acrolein promote suicidal erythrocyte death (also called eryptosis) through the induction of externalization of phosphatidylserine on the cell surface in *in vitro* experiments using human erythrocytes from healthy donors. Based on above-mentioned findings, protein-bound uremic toxins can affect erythropoiesis in the bone marrow and erythrocyte degeneration in the blood circulation as well as the process of EPO production in the kidneys to induce renal anemia.

ESAs are clinically used for many years and provide effective anemia treatment as described above. On the other hand, there are a certain number of patients (5-10% of ESAs treated patients) who are less responsive to ESAs, which is called ESA hyporesponsiveness [76, 77]. Deficiency of iron which is a component of hemoglobin and inflammation are raised for the causes of ESA hyporesponsiveness and some therapeutic approaches are under investigation[78]. In addition, protein-bound uremic toxins are also considered to be involved in that through the impairment of erythropoiesis and enhancement of erythrocyte destruction although further investigations are needed.

Finally, I summarized the effects of protein-bound uremic toxins on each process

of erythrocyte lifecycle in Figure 21. In this thesis, I have found that IS and IG suppress the EPO production which is the most important cause of renal anemia, indicating that IS and IG contribute to the progression of renal anemia in CKD patients whose blood levels of these compounds are elevated due to renal dysfunction. Furthermore, this study is the first to elucidate the detailed molecular mechanism by which the activation of AHR plays a crucial role in the suppressive effect of these protein-bound uremic toxins. Therefore, AHR activation may be one of potential therapeutic targets for treating renal anemia. These findings warrant further studies to determine the therapeutic effects of the inhibition of AHR.

Figures

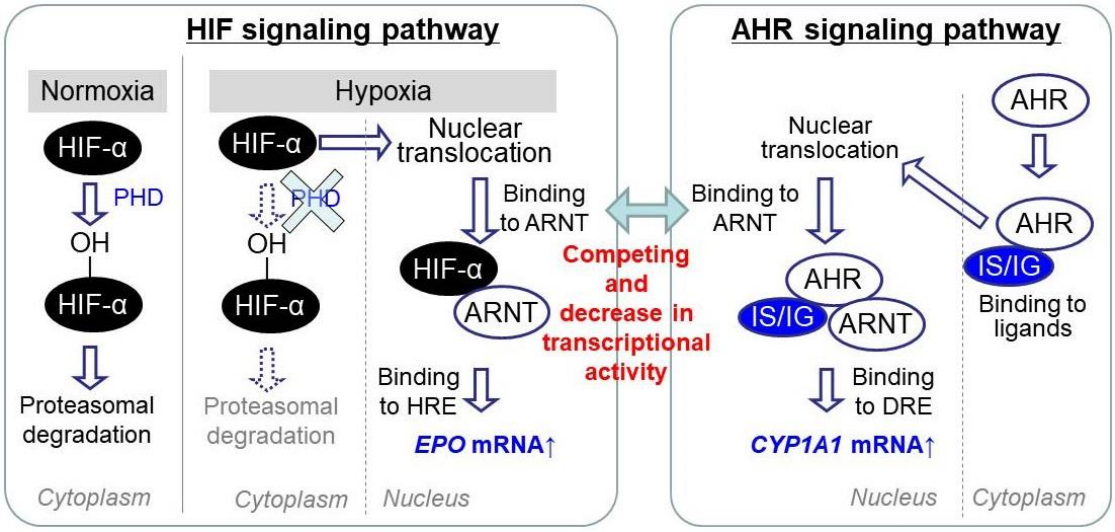

Figure 20. *The proposed mechanisms for the suppression of HIF activation by AHR ligands IS and IG.*

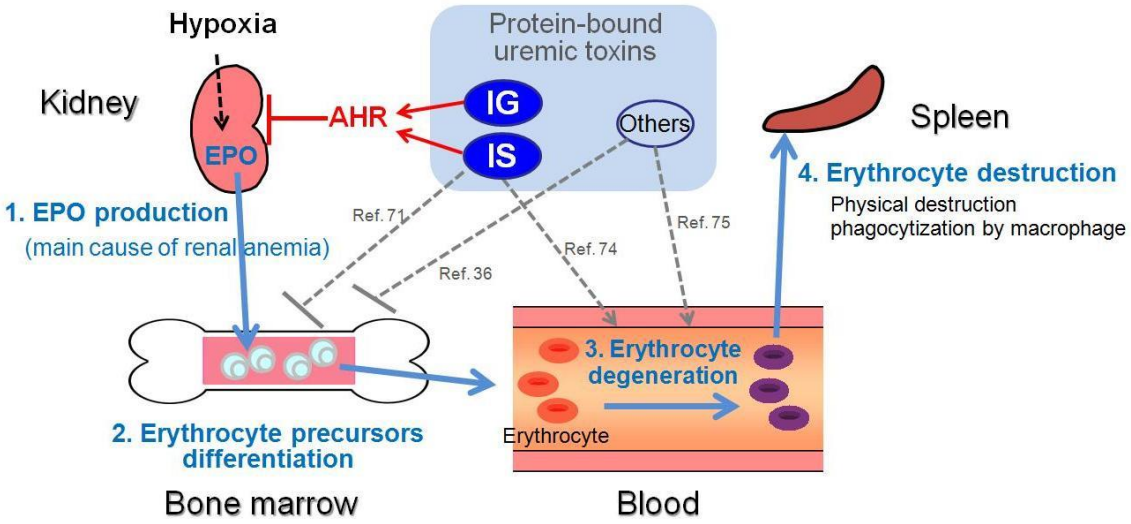

Figure 21. *Scheme of the effects of protein-bound uremic toxins on each process of erythrocyte lifecycle.*

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