Activation of aryl hydrocarbon receptor mediates suppression of hypoxia-inducible factor-dependent erythropoietin expression by indoxyl sulfate

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Asai H, Hirata J, Hirano A, Hirai K, Seki S, Watanabe-Akanuma M. Activation of aryl hydrocarbon receptor mediates suppression of hypoxia-inducible factor-dependent erythropoietin expression by indoxyl sulfate. Am J Physiol Cell Physiol 310: C142–C150, 2016. First published November 11, 2015; doi:10.1152/ajpcell.00172.2015.—Indoxyl sulfate (IS) is a representative uremic toxin that accumulates in the blood of patients with chronic kidney disease (CKD). In addition to the involvement in the progression of CKD, a recent report indicates that IS suppresses hypoxia-inducible factor (HIF)-dependent erythropoietin (EPO) production, suggesting that IS may also contribute to the progression of renal anemia. In this report, we provide evidence that IS suppresses hypoxia-inducible factor (HIF)-dependent erythropoietin (EPO) production in HepG2 cells. IS at concentrations similar to the blood levels in CKD patients suppressed hypoxia- or cobalt chloride-induced EPO mRNA expression and transcriptional activation of HIF. IS also induced AhR activation, and AhR blockade resulted in abolishment of IS-induced suppression of HIF activation. The AhR 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 AhR-ARNT complex by an increase of the AhR-ARNT complex in the nucleus, implying 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 elevation of renal EPO mRNA expression and plasma EPO concentration and strongly induced AhR activation in the liver and renal cortex tissues. Collectively, these findings prompt us to investigate the role of IS on the progression of renal anemia. IS-induced activation of AhR may be a potential therapeutic target for treating renal anemia.

RENAL DYSFUNCTION LEADS TO ACCUMULATION OF UREMIC TOXINS IN PATIENTS WITH CHRONIC KIDNEY DISEASE (CKD). Approximately 90 compounds have been reported as uremic toxins or their candidates (7, 27). Indoxyl sulfate (IS) is a representative protein-bound uremic toxin that plays a role in the progression of CKD (28). Previous studies have proposed that IS exhibits renal toxicity mainly by inducing oxidative stress on proximal tubular cells (23, 24). In addition, IS induces oxidative stress on vascular smooth muscle cells and endothelial cells (14, 26, 34). Therefore, IS is also considered to be involved in the progression of cardiovascular disease associated with CKD (2, 15).

Recently, IS has been reported to be an endogenous ligand for the aryl hydrocarbon receptor (AhR), also called the dioxin receptor (29). The AhR is a ligand-activated transcription factor and normally exists in the cytosol in an inactive form. On binding ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), this receptor translocates into the nucleus, dimerizes with the AhR nuclear translocator (ARNT), and induces the expression of several genes, including cytochrome P-450 1A1 (CYP1A1). For many years, studies of the AhR have been focused on its role in the toxic effects of dioxin-like compounds (21). Recently, however, the AhR is known to play a role in important pathophysiological functions, such as carcinogenesis (25), stem cell regulation (3, 10), and immunity (8). These findings prompt us to investigate the role of IS on the progression of renal anemia, due to inadequate erythropoietin (EPO) production from the kidneys. The expression of EPO is mediated by hypoxia-inducible factor (HIF), a heterodimeric complex composed of HIF-α subunits (such as HIF-1α and HIF-2α) and ARNT (also called HIF-1β). The expression of HIF-α is strictly controlled by cellular oxygen molecules. Under normoxia, oxygen-dependent prolyl-4 hydroxylases hydroxylate the proline residues of HIF-α, triggering the recruitment of the Von Hippel-Lindau tumor suppressor protein, a component of E3 ubiquitin ligase complex, and facilitating proteasomal degradation of HIF-α (16, 22). On the other hand, under hypoxia, HIF-α escapes from the degradation pathway, which in turn accelerates nuclear accumulation of HIF-α and dimerization with ARNT. Recently, Chiang and colleagues (5) have reported that IS suppresses hypoxia-induced HIF activation and subsequent EPO production. Their report provides important knowledge for the elucidation of the role of IS on the progression of renal anemia, but the mechanism by which IS suppresses HIF activation remains unclear.

The purpose of the present study was to determine whether AhR contributes to the suppression of HIF activation by IS. The results indicated that IS at concentrations similar to blood levels in CKD patients suppressed the expression of EPO mRNA and the transcriptional activation of HIF. Furthermore, IS strongly induced the activation of AhR, and AhR blockade resulted in abolishment of the effect of IS in suppressing HIF activation. These findings demonstrate that AhR plays an indispensable role in the suppressive effects of IS on hypoxia-induced HIF activation and subsequent EPO production.

MATERIALS AND METHODS

Animal experiments. Seven-week-old male Sprague Dawley (Crl: CD) rats weighing 210–260 g were purchased from Japan Charles
River (Kanagawa, Japan). Rats were housed in polycarbonate cages in an animal room under controlled illumination (12:12-h light-dark cycle), temperature (22 ± 2°C), and humidity (55 ± 10%). All experimental procedures were approved by the Committee of Ethics on Animal Experiments at Kureha Corporation (Tokyo, Japan) and were conducted in accordance with the guidelines of Kureha Corporation. After acclimatization for at least 6 days, the rats were fasted for 20 h before the start of experiments. In the bleeding experiments, 1 ml blood/100 g body wt was drawn from the cervical vein of rats under inhaled isoflurane anesthesia to induce physiological EPO production. After recovery from anesthesia, the rats were administered 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 to increase the blood concentration of IS. Thereafter, blood was collected 3, 9, and 24 h after indole administration, and rats were euthanized after the last blood sampling. For the analysis of EPO mRNA expression, rats were euthanized 9 h after indole administration, and the liver and renal cortex tissues were collected. In the experiments on CYP1A1 mRNA expression and AhR protein analysis, healthy rats were administered indole as described above and euthanized 3, 6, or 9 h later. The liver and renal cortex tissues were collected, snap frozen in liquid nitrogen, and stored at −80°C until RNA isolation and nuclear protein extraction.

Measurement of plasma EPO and IS concentration. Plasma EPO concentrations were measured using a Mouse/Rat EPO Serum/Plasma kit (MA6000; Meso Scale Discovery, Rockville, MD), 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. (19).

Cell culture, induction of hypoxic response, and IS treatment. The human hepatoma cell line HepG2 was purchased from DS Pharma Biomedical (Osaka, Japan). The cell line retains the ability to express EPO in response to hypoxic culture or treatment with hypoxia-mimicking agents such as cobalt chloride (11). The culture medium was MEM (Sigma-Aldrich, St. Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 1% nonessential amino acid solution (Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Tokyo, Japan), except in transfection experiments in which antibiotic-free medium was used. Cells were cultured at 37°C in a 5% CO2 humidified incubator, unless otherwise indicated. For induction of cellular hypoxic response, cells were treated with 50 µM cobalt chloride (Wako Pure Chemical Industries). Alternatively, cells were cultured under hypoxic condition using an AnaeroPack System (Mitsubishi (Wako Pure Chemical Industries). The final concentration of DMSO in the medium was 0.2% in 1 h and providing a 5% CO2 atmosphere without changing medium pH (18, 31), for periods indicated in the Figs. 1–8 legends. IS 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). 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 95°C for 3 s, 60°C for 30 s). Values for the mRNA were normalized to hypoxanthine phosphoribosyltransferase mRNA expression for human genes and GAPDH mRNA expression for rat genes. Analyses were conducted with a Thermal Cycler Dice Real Time System (Takara Bio). The sequences of primers used are listed in Table 1.

Transient transfection and hypoxia response element-luciferase assay. The luciferase reporter plasmid for hypoxia response element (HRE) [pGL4.42 (luc2P/HRE/Hygro) Vector] and control plasmid [pGL4.74 (hRluc/TK) Vector] were purchased from Promega (Madison, WI). HepG2 cells (1 × 10⁴/well) were seeded into 96-well plates and cultured overnight. After cotransfection with both the HRE-luciferase and control plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, Tokyo, Japan) and subsequent cultivation for 24 h, cells were treated with IS and cultured with cobalt chloride or under hypoxic condition, as described above. Cells were then washed with ice-cold phosphate-buffered saline, and cellular luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega) with a Lumat LB9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). Data are presented as the ratio of Photoris pyralis luciferase activity/Renilla reniformis luciferase activity.

In some experiments, the AhR antagonist CH-223191 dissolved in DMSO was added at the same time of IS treatment. In experiments using small interfering RNA (siRNA), cells were transfected simultaneously with the luciferase reporter plasmid and AhR siRNA (ON-TARGETPlus siRNA, human AhR, catalog no. J-004900-07) or nontargeting siRNA (ON-TARGETPlus Control siRNA, no. 1, catalog no. D-001810-01-05; both from Thermo Fisher Scientific, Kanagawa, Japan). For the assessment of the effect of the siRNAs, siRNA-transfected cells were harvested at the start of IS treatment.

Table 1. List of primer pairs used in quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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<tbody>
<tr>
<td>Human erythropoietin</td>
<td>AGCCCAGAAGGAGCACTCT</td>
<td>GAAAGTGTCACAGTAGTTT</td>
</tr>
<tr>
<td>Human CYP1A1</td>
<td>TCCCTGTGGTCCTCCCTCA</td>
<td>TTGTTGATGTGAGGTAGTGT</td>
</tr>
<tr>
<td>Human HIF-1α</td>
<td>CATAAGGTTCGCAACTGGAAGT</td>
<td>ATTTGATGTTGAGGAGTAAGT</td>
</tr>
<tr>
<td>Human HIF-2α</td>
<td>CTGTCCTCCAGCCCAATAG</td>
<td>GTCCAGTCTGTCCAAACGT</td>
</tr>
<tr>
<td>Human HPRT</td>
<td>GACAGCTGAAAGAGGAGCAT</td>
<td>GACCTTGAGACATTTGGGA</td>
</tr>
<tr>
<td>Rat erythropoietin</td>
<td>GTCCTCAGTCTGGGTGCC</td>
<td>ATCTGCTGGAGTTGTTGGA</td>
</tr>
<tr>
<td>Rat CYP1A1</td>
<td>CCTCTTGGTAGCTGCTGCTTTGG</td>
<td>GCTGTTGGGAGATGTTGAA</td>
</tr>
<tr>
<td>Rat GAPDH</td>
<td>GATGATGGTAGAAGCCAGGAGAA</td>
<td>ACGGATCATGGGAGGTTGAGA</td>
</tr>
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CYP1A1, cytochrome P-450 1A1; HIF, hypoxia-inducible factor; HPRT, hypoxanthine Phosphoribosyltransferase.
lyzed in 1× reducing SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% 2-mercaptoethanol), and analyzed for AhR protein expression by immunoblot, as described below.

**Nuclear protein extraction and immunoprecipitation.** HepG2 cells (7.5 × 10⁶/dish) were seeded into 10-cm dishes and cultured overnight. After treatment with IS and cobalt chloride, 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 and 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 Scientific) with anti-HIF-1α rabbit polyclonal antibody, anti-HIF-2α rabbit polyclonal antibody (Medical & Biological Laboratories) or anti-lamin B1 rabbit polyclonal antibody (Cell Signaling Technology). Nuclear protein extracts were processed by immunoprecipitation procedures using a Pierce Classic IP kit (Thermo Fisher Scientific) with BSA as standard. For further extraction of ARNT-binding nuclear proteins, equal amounts (100 µg) of nuclear protein extracts were processed by immunoprecipitation procedures using a Pierce Classic IP kit (Thermo Fisher Scientific) with anti-HIF-1α/ARNT (D28F3) XP Rabbit MAb (Cell Signaling Technology, Danvers, MA), according to the manufacturer’s instructions. Proteins bound to the antibody were then eluted with an acid-based buffer.

**Immunoblot analysis.** Cell lysates, nuclear protein extracts, and immunoprecipitated nuclear proteins were mixed with reducing SDS sample buffer and heated for 3 min at 100°C. The samples were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (GE Healthcare). The membranes were blocked with 3% skim milk solution and treated with one of the following primary antibodies: anti-HIF-1α rabbit polyclonal antibody, anti-HIF-2α rabbit polyclonal antibody (Cell Signaling Technology), anti-AhR mouse MAb (A-3; Santa Cruz Biotechnology, Dallas, TX), anti-lamin B1 rabbit polyclonal antibody (Medical & Biological Laboratories, Aichi, Japan), and anti-α-tubulin rabbit polyclonal antibody (Medical & Biological Laboratories). 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 LightCapture AE-6981 (Atto, Tokyo, Japan).

**Statistical analysis.** Statistical significance was tested by two-tailed unpaired t-test (single comparisons) using Excel 2010 (Microsoft, Tokyo, Japan), or by Dunnett test or Tukey-Kramer honestly significant difference test (multiple comparisons) using JMP (version 10.0.0, SAS Institute, Cary, NC). Differences were considered statistically significant for P values <0.05.

**RESULTS**

**IS suppresses hypoxia-induced elevation of EPO mRNA expression in HepG2 cells.** IS at concentrations up to 1,500 µM had no influence on viability of HepG2 cells, in both the presence and absence of cobalt chloride (data not shown). Thus the highest concentration of IS was set at 1,500 µM in subsequent experiments.

In the absence of hypoxic stimulation, IS up to 1,500 µM had no effect on EPO mRNA expression (Fig. 1A). Under hypoxic culture conditions (O₂ < 1%), EPO mRNA expression was elevated significantly by 2.8-fold, and this elevation was significantly suppressed by IS at 100 µM or higher (Fig. 1B). Similarly, IS significantly suppressed the increase of EPO mRNA expression induced by treatment with cobalt chloride (Fig. 1C).

**Oral administration of indole increases plasma IS concentration and suppresses bleeding-induced elevation of plasma EPO concentration and renal EPO mRNA expression in rats.** A single oral dose of indole at 100 mg/kg to rats caused marked increases in plasma IS concentration (5 ± 1 µM in vehicle-treated rats; 127 ± 23, 52 ± 19, and 29 ± 9 µM at 3, 6, and 9 h, respectively, after administration in indole-treated rats).

In the bleeding experiments in which 1 ml blood/100 g body wt was drawn from each rat, mean plasma IS concentration was elevated to 140 µM by a single oral dose of indole at 100 mg/kg, whereas IS concentration was <10 µM throughout the experiment in the vehicle group (Fig. 2A). Plasma EPO concentration increased gradually over time after blood withdrawal in the vehicle group, but the increase was significantly suppressed by administration of indole (Fig. 2B). Blood withdrawal also induced EPO mRNA expression in the renal cortex but not in the liver, and the induction was significantly suppressed by administration of indole (Fig. 2C).

**IS suppresses hypoxia-induced transcriptional activation of HIF in HepG2 cells.** Transcriptional activity of HIF was assayed by measuring HRE-luciferase activity in HepG2 cells. In the absence of hypoxic stimulation, IS even at 500 µM had no effect on EPO mRNA expression (Fig. 1A). Hypoxic stimulation (O₂ < 1%) increased HRE-luciferase activity signifi-

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**Fig. 1.** Effect of indoxyl sulfate (IS) on hypoxia-induced erythropoietin (EPO) mRNA expression in HepG2 cells. Serum-starved cells were treated with IS under normoxic condition (A), hypoxic condition (O₂ < 1%; B), or in the presence of 50 µM cobalt chloride (CoCl₂; C) for 6 h. CoCl₂ was added 1 h after IS treatment. EPO mRNA expression was then measured by quantitative real-time RT-PCR. Values are means ± SD of 3–4 independent experiments. **P < 0.01 and ***P < 0.001 (unpaired t-test). **P < 0.01 and ***P < 0.001 (Dunnett test).
cantly by 6.50-fold, and this increase was significantly suppressed by IS at 100 μM or higher (Fig. 3B). Furthermore, in the case of cobalt chloride treatment, the 5.24-fold increase in HRE-luciferase activity was significantly suppressed by IS at 20 μM or higher (Fig. 3C). Since transcriptional activation of HIF is induced by nuclear accumulation of HIF-α subunits, the amounts of HIF-1α and HIF-2α in nuclear extracts were measured. Cobalt chloride treatment increased nuclear accumulation of both HIF-1α and HIF-2α, and this increase was suppressed by IS (Fig. 3D).

To examine the possibility that the suppression by IS is due to inhibition of endogenous expression of HIF-α subunits, the effect of IS on mRNA expression of HIF-α subunits was examined. IS up to 500 μM had no effect on HIF-2α mRNA expression in the presence and absence of cobalt chloride (data not shown). IS also had no effect on HIF-1α mRNA expression in the absence of cobalt chloride, but IS at 500 μM induced a slight increase in HIF-1α mRNA expression in the presence of cobalt chloride (Fig. 3E). Hence, IS did not decrease HIF-1α or HIF-2α mRNA expression. Consequently, IS suppressed nuclear accumulation of HIF-1α and HIF-2α by mechanisms other than inhibition of endogenous expression.

Inactivation of AhR abolishes the suppressive effect of IS on HIF activation and EPO mRNA expression in HepG2 cells. In the absence of hypoxic stimulation, HRE-luciferase activity was not affected by treatment with IS, an AhR antagonist CH-223191, or both (Fig. 4A). The increase in HRE-luciferase activity induced by hypoxic culture (O₂ < 1%) was significantly suppressed by IS (100 μM), and IS-induced suppression was completely abolished by cotreatment with CH-223191 (1 μM), whereas CH-223191 alone had no influence on hypoxia-induced HIF activation (Fig. 4B). Similarly, the increase in HRE-luciferase activity induced by cobalt chloride was significantly suppressed by IS (100 μM) and IS-induced suppression was abolished by cotreatment with CH-223191 in a concentration-dependent manner (Fig. 4C).

In the experiments using siRNA, transfection of AhR-siRNA inhibited cellular expression of AhR protein, while transfection of nontargeting siRNA had no effect (Fig. 4D). Cobalt chloride induced significant increases in HRE-luciferase activity in all cells, and this increase was suppressed by IS (100 μM) in nontargeting siRNA-transfected cells, but not in AhR-siRNA-transfected cells (Fig. 4E). Hence, AhR-siRNA transfection abolished the suppression of cobalt chloride-induced HIF activation by IS.

Since the HRE-luciferase assay measures HIF activation but not EPO expression, the effect of AhR inactivation on the suppression of EPO mRNA expression by IS was further examined. The elevation in EPO mRNA expression induced by hypoxic culture (O₂ < 1%) was significantly suppressed by IS (500 μM), and the suppression was abolished by cotreatment with CH-223191 (1 μM), while CH-223191 alone had no effect on hypoxia-induced EPO mRNA expression (Fig. 4F).

TCDD also suppresses hypoxia-induced transcriptional activation of HIF in HepG2 cells. Treatment with TCDD and/or CH-223191 had no effect on HRE-luciferase activity (Fig. 5, left). In contrast, cobalt chloride-induced increase in HRE-luciferase activity was significantly suppressed by treatment with TCDD (10 nM), and this suppression was completely abolished by cotreatment with CH-223191 (Fig. 5, right). Furthermore, nuclear accumulation of HIF-1α and HIF-2α was suppressed by TCDD, as well as by IS (Fig. 3D).

IS induces activation of AhR in both HepG2 cells and rats. In HepG2 cells, IS significantly induced the expression of...
CYP1A1 mRNA, one of the major genes regulated by AhR, by 50-fold even at 100 μM (Fig. 6A). Furthermore, IS increased the amount of AhR in nuclear protein extract in a concentration-dependent manner (Fig. 6B).

In rats, administration of indole that caused marked increases in plasma IS concentration (as described above) induced significant CYP1A1 mRNA expression in both the liver and renal cortex tissues compared with vehicle-treated rats. Particularly in the renal cortex tissue, the increase was 726-fold at 3 h after indole administration and was still 16-fold at 9 h, although there was no significant difference (Fig. 7A). In the liver, the amount of AhR in nuclear protein extract increased significantly by administration of indole (Fig. 7B). Meanwhile, in the renal cortex, no AhR was detected in the nuclear protein extracts of all samples, due to the small amount of this protein (data not shown).

IS increases the amount of AhR-ARNT complex but decreases HIF-α-ARNT complex in the nucleus of cobalt chloride-treated HepG2 cells. To assess the amounts of both AhR-ARNT and HIF-α-ARNT complexes in the nucleus, ARNT-binding proteins were immunoprecipitated from the nuclear protein extracts of cobalt chloride-treated HepG2 cells and the expressions of AhR, HIF-1α, and HIF-2α were detected by immunoblot. The amount of AhR bound to ARNT increased by IS treatment in a concentration-dependent manner. In contrast, the amounts of HIF-1α and HIF-2α bound to ARNT decreased in inverse proportion to the increase in amount of the AhR-ARNT complex (Fig. 8).

DISCUSSION

Renal anemia is common among CKD patients, which is mainly caused by inadequate EPO production due to renal tissue damage. Renal dysfunction also leads to accumulation of uremic toxins because of reduced excretion from the kidneys. Several reports indicate that the uremic toxins play a role in renal anemia, as well as the progression of renal diseases (20, 32). Recently, Chiang et al. (5) reported that IS, a representative protein-bound uremic toxin, suppressed hypoxia-induced HIF activation and subsequent EPO production. In the present study, we investigated the detailed mechanism of the suppressive effect of IS using EPO-producing HepG2 cells and rats.
First, we investigated whether IS at clinically relevant concentrations (109 ± 61 μM) in CKD patients reported previously (7) suppressed hypoxia-induced HIF activation and subsequent EPO production in vitro. We found that hypoxia- or cobalt chloride-induced EPO mRNA expression was suppressed by IS at 100 μM or higher in HepG2 cells. Furthermore, HRE-luciferase reporter assay revealed that HIF 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 demonstrate that IS suppresses hypoxia-induced HIF activation and EPO expression at concentrations similar to the blood levels of CKD patients.

Chiang et al. (5) also reported that oral administration of indole, a metabolic precursor of IS, suppressed EPO production induced by subcutaneous administration of cobalt chloride in rats, suggesting that IS suppresses cobalt chloride-induced EPO production in vivo. Although their report provides in vivo evidence that IS suppresses EPO production under a hypoxia mimetic state, it remains unclear whether their results reflect the real effect of IS under a hypoxic condition. Thus we investigated the effect of indole administration on bleeding-induced physiological EPO induction in rats. We found that a single oral dose of indole at 100 mg/kg increased plasma IS concentration to 140 μM, which was similar to the concentration used in the above-mentioned in vitro experiments, and the indole dose suppressed the increase in EPO mRNA expression in renal cortex tissue and EPO concentration in plasma. These results suggest that IS at concentrations similar to plasma levels of CKD patients suppresses hypoxia-induced EPO production in the kidneys in vivo.

Schroeder et al. (29) reported that IS was an endogenous ligand for AhR. The AhR is a ligand-dependent transcrip-
tion factor and belongs to the family of basic helix loop helix and Per-Arnt-Sim domain proteins that also includes HIF proteins (30). Therefore, we investigated the involvement of AhR on the suppressive effect of IS on HIF activation in HepG2 cells. We found that inactivation of AhR by either the pharmacological AhR antagonist CH-223191 or AhR-siRNA abolished the suppressive effect of IS on HIF activation. Furthermore, the AhR antagonist CH-223191 inhibited IS-induced suppression of EPO mRNA expression. Since AhR is a receptor for dioxin-like compounds (21), we further examined whether the potent AhR agonist TCDD has similar effect as IS. We found that TCDD strongly suppressed cobalt chloride-induced HIF activation, and the suppression was completely abolished by CH-223191. These results demonstrate that the AhR plays an indispensable role in the suppressive effect of IS. Moreover, nuclear accumulation of HIF-α, an important step for transcriptional activation of HIF (12, 17), was suppressed by IS as well as TCDD, indicating that inhibition of nuclear accumulation of HIF-α subunits contributes to IS-induced suppression of HIF activation and subsequent EPO production.

We next examined whether IS activated AhR in both in vitro and in vivo experiments. As reported by Schroeder et al. (29), IS strongly induced the expression of CYP1A1 mRNA in HepG2 cells. CYP1A1 is one of the major genes regulated by AhR, but the specificity of CYP1A1 induction in AhR activation is questionable (13). For this reason, we further examined the nuclear translocation of AhR in HepG2 cells and confirmed the increase by IS at 20 μM or higher. Furthermore, we found 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 of rats. These results suggest that IS induces AhR activation in vivo as well as in vitro.

As mentioned above, IS suppressed the transcriptional activation of HIF 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. We thus investigated the amounts of HIF-α subunits and AhR that form heterodimeric complexes with ARNT in the nucleus by co-immunoprecipitation using anti-ARNT MAb. As IS concentration increased, the amount of HIF-α-ARNT complex in the nucleus decreased, whereas the amount of AhR-ARNT complex increased. Chilov et al. (6) reported that accumulation of HIF-1α in the nucleus of hypoxic cells was independent of the presence of ARNT, but heterodimerization was required for stable association within the nuclear compartment. Therefore, IS-induced inhibition of nuclear accumulation of HIF-α subunits could be caused by the inhibition of heterodimerization between HIF-α and ARNT. Furthermore, Chan et al. (4) reported that AhR competitively inhibited the binding between HIF-1α and ARNT, and vice versa. Although this report does not refer to HIF-2α, based on the structural similarity (9), it is assumed that HIF-2α has the same property as HIF-1α. Therefore, the present results suggest that, in the presence of IS, increased nuclear translocation of AhR inhibits the formation of HIF-α-ARNT complex, which in turn sup-
presses the transcriptional activation of HIF and subsequent EPO production.

The results of the present study indicate that IS-induced AhR activation may be one of the molecular mechanisms causing the progression of renal anemia. On the other hand, apart from IS, some other protein-bound uremic toxins have been reported to play a role in renal anemia by mechanisms other than EPO production. For example, polyamines inhibit erythroid cell differentiation through inhibition of iron incorporation into newly synthesized heme (19), and acrolein promotes suicidal erythrocyte death through induction of cell membrane scrambling and shrinkage (1). A clinical study also suggests that p-cresyl sulfate may contribute to renal anemia (33). Although further studies to elucidate the molecular mechanism of the effect of these uremic toxins are needed, both IS and these uremic toxins could be involved in the progression of renal anemia.

Collectively, the results of this study demonstrate that IS at concentrations similar to the blood IS levels in CKD patients suppresses HIF-dependent EPO production both in vitro and in vivo. Although further investigations in CKD model animals and patients are required, our data suggest that IS contributes to the progression of renal anemia in CKD patients whose blood IS levels are elevated due to renal dysfunction. Furthermore, this study is the first to elucidate the detailed mechanism by which the activation of AhR plays an indispensable role in the suppressive effect of IS. Hence, IS-induced AhR activation may be one of potential therapeutic targets for treating renal anemia.

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DISCLOSURES

All authors are employed by Kureha Corporation.
AUTHOR CONTRIBUTIONS


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