Indoxyl sulfate suppresses hepatic fetuin-A expression via the aryl hydrocarbon receptor in HepG2 cells

Akinobu Ochi1, Katsuhito Mori1, Shinya Nakatani1, Masanori Emoto1, Tomoaki Morioka1, Koka Motoyama1, Shinya Fukumoto1, Yasuo Imanishi1, Tetsuo Shoji2, Eiji Ishimura1 and Masaaki Inaba1

1Department of Metabolism, Endocrinology and Molecular Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan
and 2Department of Geriatrics and Vascular Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan

Correspondence and offprint requests to: Katsuhito Mori; E-mail: ktmori@med.osaka-cu.ac.jp

ABSTRACT

Background. Fetuin-A is a liver-derived circulating protein that has potent calcification-inhibitory activity. Uraemic patients exhibit decreased serum fetuin-A levels, increased vascular calcification and elevated cardiovascular mortality. Because the mechanisms for fetuin-A deficiency are unknown, we hypothesized that some uraemic toxins suppressed hepatic fetuin-A production, which resulted in accelerated vascular calcification and poor outcome. Among these potential candidates, indoxyl sulfate (IS) has highly toxic properties.

Methods. We examined the direct effects of IS on hepatic fetuin-A expression using the human hepatoma HepG2 cell line.

Results. IS, but not p-cresyl sulfate, suppressed the mRNA and protein expression of fetuin-A in a dose- and time-dependent manner. As reported previously, IS stimulated p38 MAPK phosphorylation and reactive oxygen species (ROS) production, although the knockdown of p38 and inhibition of ROS generation had no effect on IS-induced fetuin-A suppression. Then, because IS is a potent endogenous ligand of the aryl hydrocarbon receptor (AhR), we assessed whether IS suppresses fetuin-A production via AhR. The knockdown of AhR prevented IS-induced fetuin-A suppression. However, some attention should be paid to no effect of IS on fetuin-A expression in mouse and human primary cultured hepatocytes.

Conclusions. These findings suggest that IS could suppress hepatic fetuin-A expression by activating AhR, suggesting a relationship between uraemia and fetuin-A deficiency.

Keywords: AHSG, fetuin-A, hepatocyte, indoxyl sulfate

INTRODUCTION

Fetuin-A, also known as alpha-2-HS-glycoprotein (AHSG), is an abundant circulating protein that is produced mainly by the liver. Among its multiple functions in human physiology and pathology [1, 2], one of its most prominent features is a potent calcification-inhibitory capacity [3–5]. Fetuin-A seems to prevent ectopic calcification by forming water-soluble mineral complexes that mainly comprise calcium, phosphate and fetuin-A. These complexes, termed calciprotein particles (CPPs) [6, 7] or fetuin-A mineral complexes (FMCs) [8, 9], can buffer mineral ion super-saturation in the circulation [10, 11].

Uraemic patients have decreased serum fetuin-A concentrations, increased vascular calcification and poor prognosis. Therefore, fetuin-A is assumed to play a role in the high mortality of dialysis patients. Ketteler et al. [12] were the first to report that low serum fetuin-A levels were a novel predictor of all-cause and cardiovascular mortality in haemodialysis patients. Subsequent studies confirmed the association between fetuin-A deficiency and poor outcome not only in patients...
undergoing haemodialysis but also in those undergoing peritoneal dialysis [13–15]. However, the mechanisms by which fetuin-A levels are decreased in uraemic patients are not fully understood.

Recently, Herrmann et al. [16] reported that fetuin-A-containing CPPs were cleared by the reticuloendothelial system. It is possible that the profound calcification stress in uraemia lowers serum fetuin-A levels by causing rapid clearance of CPPs, although direct evidence for this is lacking. In contrast, the hepatic production of fetuin-A may be suppressed during uraemia. Uraemic patients generally have protein energy wasting (PEW) syndrome, which has been associated with malnutrition, persistent inflammation, dialysis procedure, hormonal derangements and comorbidities [17]. PEW-related inflammation and/or malnutrition leads to a decline in albumin that, like fetuin-A, is a negative phase protein. Therefore, fetuin-A deficiency in uraemia may merely reflect the consequence of PEW syndrome [5], although no previous studies have explored this.

Uraemia or the advanced stages of chronic kidney disease (CKD) are characterized by the accumulation of uraemic toxins [18, 19]. Among these, indoxyl sulphate (IS) has captured attention because of its relatively highly toxic properties [18, 19]. Dietary-derived tryptophan is metabolized into indole by the intestinal flora, and the subsequently absorbed indole is further converted into IS in the liver. Because IS is excreted into the urine via the kidneys, it accumulates gradually concurrent with the development of renal dysfunction. Serum IS levels are extremely elevated in uraemic patients who require treatment with dialysis. Haemodialysis cannot normalize serum IS levels because of a very low elimination efficiency during dialysis due to its high binding affinity for albumin. The accumulated IS can induce oxidative stress in various tissues and organs, including the cardiovascular system [18, 19]. In addition, because IS is a known endogenous ligand for the aryl hydrocarbon receptor (AhR) [20–23], its toxicity may be exerted partly via an AhR-dependent mechanism.

In the current study, we tested the hypothesis that accumulated IS suppresses hepatic fetuin-A production using cultured human hepatoma HepG2 cells.

**MATERIALS AND METHODS**

**Reagents**

All cell culture plastic wares were purchased from Becton-Dickinson (NJ, USA). IS potassium salt, human serum albumin, N-acetyl cysteine (NAC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were obtained from Sigma-Aldrich (MO, USA). p-Cresyl sulphate (PCS) was obtained from Kureha Corporation (Tokyo, Japan). Potassium chloride (KCl) was obtained from WAKO (Osaka, Japan).

**Cell culture**

Human hepatocellular carcinoma HepG2 cells were purchased from American Type Culture Collection (ATCC, VA, USA). Cells were cultured and maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, CA, USA) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen) in a humidified atmosphere containing 5% CO₂ at 37°C. The culture media were replaced every 2 days. After the cultures reached confluence, cells were serum-starved overnight and subsequently incubated for 0, 6, 12, 18 or 24 h with different doses (0, 0.25, 0.5 and 1 mM) of IS. Similarly, cells were treated with or without 0.53 or 1.06 mM PCS or 30 nM TCDD for 24 h. NAC, 4% human serum albumin or KCl was added simultaneously with IS as indicated. Mouse and human primary cultured hepatocytes were purchased from COSMO BIO (HPC03P) (Tokyo, Japan) and ScienCell Research Laboratories (no. 5200) (CA, USA), respectively. Cells were cultured and maintained according to the manufacturer’s instructions. Cells were then serum- and growth factor-starved overnight and subsequently incubated for 24 h with different doses (0, 0.5 and 1 mM) of IS.

**Gene knockdown using small-interfering RNAs**

HepG2 cells were cultured in DMEM with 10% FBS for 2 days until they reached a confluency of approximately 70%. The cells were then harvested, re-suspended and transfected with pre-designed small-interfering RNA (siRNA) against human AhR or human p38 (Thermo Scientific, MI, USA) using an Amaxa Nucleofector 1 electroporation system (Amaxa Biosystems), according to the manufacturer’s instructions. Silencer Select Negative Control no. 1 siRNA (Applied Biosystems, CA, USA) was used as a negative control. After 24 h, the cells were serum-starved overnight and then treated with or without 1 mM IS for 24 h.

**RNA isolation and real-time quantitative RT–PCR**

RNA was isolated from cells using TRIzol reagent (Invitrogen), and 2 μg was then used to synthesize cDNA by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. The expression of human Ahsg and 18S ribosomal RNA were measured quantitatively using TaqMan Real-Time PCR (Applied Biosystems). Ready-to-use primers for human fetuin-A/Ahsg (Hs00155659) and 18S ribosomal RNA (4319413E) were purchased from Applied Biosystems (TaqMan Gene Expression Assays) and were used according to the manufacturer’s instructions. A cycle threshold (Ct) value was measured for each sample, and the mRNA expression levels were determined using a comparative Ct method with 18S ribosomal RNA as the endogenous control (Applied Biosystems) [24].

**Western blotting**

After each treatment, cells were washed twice with ice-cold buffer (137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mM Na₂VO₄ and 20 mM Tris–HCl at pH 7.6) and lysed in the same buffer supplemented with 1% Nonidet P-40, 10% glycerol, 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM Na₂VO₄, 2 mM phenylmethylsulfonyl fluoride and 8 μg/mL leupeptin. After the removal of insoluble materials by centrifugation, the protein concentrations were determined using BCA protein assays (Thermo Scientific). Cell lysates (40 μg protein per lane) were mixed with sample-loading buffer and subjected to 10% SDSPAGE. The gels were then equilibrated in 50 mM Tris–HCl buffer (pH 6.8), 20% glycerol, 5% SDS, 5% mercaptoethanol, 100 mM DTT and 0.002% bromophenol blue for 30 min at 37°C, followed by electrophoresis. The separated proteins were then transferred to a nitrocellulose membrane and blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20. The membrane was incubated with primary antibodies, and the immune complexes were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence reagents.
and separated on 10% SDS-PAGE gels. The proteins were then transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) and incubated with antibodies against human fetuin-A (R&D Systems, MN, USA), mouse fetuin-A (Santa Cruz Biotechnology Inc., CA, USA), human AhR (Santa Cruz Biotechnology Inc.), ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, p38, phospho-p38, GAPDH and α-tubulin (all from Cell Signalling Technology, Danvers, NA, USA). Each antibody was diluted using the dilution buffer (154 mM NaCl, 7.7 mM sodium azide, 50 mM Tris at pH 7.8 with 5% BSA) (dilution ratio was 1 : 1000) except antibodies against fetuin-A and AhR. The final concentration of anti-human fetuin-A antibody was 0.2 µg/mL. The dilution ratio of anti-AhR antibody and anti-mouse fetuin-A antibody was 1 : 200. Protein bands were identified using ECL (GE Healthcare, NJ, USA) and were quantified using a densitometer (Lumino Shot™ 400Jr; TAKARA Bio Inc., Shiga, Japan) [24, 25].

Transfections and Dual Luciferase Reporter Assay

HepG2 cells were seeded into 48-well plates and transfected using Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions after they reached ~70% confluence. Cells were transfected with 150 ng of a PGL4.43[luc2P/XRE/Hygro] (Promega) firefly luciferase reporter plasmid containing or lacking the XRE sequence (Promega). Cells were also co-transfected with 5.0 ng of a pRL-null (Promega) Renilla luciferase reporter plasmid as an internal control. After co-transfection, cells were serum-starved overnight and then treated with or without 1 mM IS for 24 h. Then, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Wallac ARVO SX 1420 Multi-label Counter; PerkinElmer Inc., MA, USA) according to the manufacturer’s instructions. Results were adjusted to Renilla luciferase activity.

Intracellular ROS production

HepG2 cells were seeded into 96-well dark plates. On reaching ~70% confluence, cells were treated using DCF-DA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK) in the presence of 10% FBS according to the manufacturer’s instructions. After pretreatment with DCF-DA, cells were serum-starved in phenol red-free DMEM with or without 1 mM IS for 24 h. The fluorescence resulting from ROS production within the cells at different periods (0, 4, 8, 16 and 24 h) using luminometer (Wallac ARVO SX 1420 Multi-label counter) was measured at various periods. The level of fluorescence intensity was adjusted to the Luciferase Reporter Assay System (Promega) and a luminometer (Wallac ARVO SX 1420 Multi-label Counter; PerkinElmer Inc., MA, USA) according to the manufacturer’s instructions [26].

Statistical analysis

Each experiment was repeated at least three times to confirm the results. All data are presented as means ± standard deviation (SD). Statistically significant differences among more than three groups were analysed using ANOVA followed by Dunnett’s or Scheffe’s post hoc tests. All analyses were performed using StatView 5 (SAS Institute Inc., Cary, NC, USA) designed for Windows. The level of statistical significance was set at P < 0.05.

RESULTS

Inhibitory effects of IS on both the mRNA and protein expression of fetuin-A in hepatocytes

To investigate whether IS could suppress fetuin-A expression directly in hepatocytes, we cultured HepG2 cells with or without IS. Treatment with 1 mM IS for 24 h down-regulated both the mRNA (by 38.5%; Figure 1C) and protein (by 38.7%; Figure 1B1 and B2) expression of fetuin-A in a dose-dependent manner. In addition, treating HepG2 cells with 1 mM IS suppressed fetuin-A protein expression in a time-dependent manner (42.5% decrease in 24 h; Figure 1A1 and A2). Because IS has a high affinity for albumin, we also examined the effects of IS on fetuin-A expression in the presence of 4% human serum albumin [27]. The addition of 4% albumin to the culture system did not affect IS-induced suppressed fetuin-A expression significantly (Figure 2A1 and A2). Furthermore, because IS is potassium salt, we examined whether KCl could affect fetuin-A expression as a control. No effect of KCl was observed in this system (Figure 2B1 and B2).

Effect of IS on fetuin-A suppression compared with the uraemic toxin p-cresyl sulfate

To confirm whether the inhibitory effect of IS on fetuin-A expression was not common among uraemic toxins, we compared its effects to those of the uraemic toxin PCS. As expected, fetuin-A expression was suppressed significantly by treatment with 1 mM IS for 24 h (Figure 2C1 and C2). In contrast, 0.53 or 1.06 mM of PCS did not significantly affect fetuin-A expression in HepG2 cells (Figure 2C1 and C2). Similarly, we examined the combined effects of IS and PCS on fetuin-A expression. No synergistic effects of IS and PCS on fetuin-A suppression were found (Figure 2D1 and D2). These findings suggest that the inhibitory effects of IS on fetuin-A expression may not be common among uraemic toxins.

No involvement of MAPK signalling in the IS-induced suppression of fetuin-A

To evaluate the intracellular signalling pathways that are involved in the IS-induced suppression of fetuin-A, we examined various mitogen-activated protein kinase (MAPK) pathways including ERK1/2, JNK and p38. The phosphorylation of ERK1/2 and JNK was unaffected by treatment with 1 mM IS for 24 h (Figure 3A). In contrast, IS stimulated p38 phosphorylation significantly in HepG2 cells. To investigate the involvement of p38 on the effects of IS on fetuin-A expression, we next knocked down p38 expression in HepG2 cells. Transfecting HepG2 cells with siRNA specific for p38 decreased p38 protein expression significantly (Figure 3B). However, the IS-induced suppression of fetuin-A was unaffected by knocking down p38 (Figure 3B). Therefore, various MAPK pathways, including p38, may not be involved in the IS-induced suppression of fetuin-A.

No effect of IS-induced ROS on fetuin-A expression

Because IS induces oxidative stress in various cells [26–28], we measured the fluorescence intensity of the ROS probe.
DCF-DA in HepG2 cells in the presence or absence of 1 mM IS with or without 10 mM of the antioxidant NAC. As expected, IS induced ROS production in HepG2 cells, which was abolished completely in the presence of 10 mM NAC (Figure 4A). We next used these conditions to examine whether IS suppressed fetuin-A expression via ROS production. NAC had no effect on the IS-induced suppression of fetuin-A expression (Figure 4B1 and B2). Therefore, although IS clearly caused oxidative stress in HepG2 cells, the generated ROS may not be involved in IS-induced fetuin-A suppression.

**Requirement for AhR in an IS-activated reporter gene assay**

The AhR is a xenobiotic receptor for TCDD; dioxin [29, 30]. The binding of TCDD to AhR causes an interaction with xenobiotic-responsive element (XRE) sequences in the promoter regions of a wide range of target genes, which results in diverse toxic effects in a variety of species and tissues [29]. Recent studies demonstrated that IS could act as an endogenous AhR agonist [20–23]. Therefore, we hypothesized that IS could activate an AhR-dependent pathway to suppress fetuin-A expression in HepG2 cells.
fetuin-A suppression. To confirm this, we first utilized HepG2 cells that transiently expressed an XRE-coupled luciferase reporter. As expected, the addition of 30 nM TCDD resulted in XRE-coupled reporter activity (Figure 5A1). Interestingly, significantly increased luciferase activity was observed in the presence of 1 mM IS (Figure 5A1). We next examined whether

**FIGURE 2**: Effects of 4% human serum albumin or KCl or PCS on fetuin-A expression in HepG2 cells. (A) HepG2 cells were treated with or without 1 mM IS in the absence or presence of 4% human serum albumin for 24 h. (A1) Western blots of cell lysates isolated from HepG2 cells were probed with fetuin-A or α-tubulin antibodies. (A2) Fetuin-A protein levels were determined using α-tubulin as an endogenous reference, and fetuin-A levels without IS in the absence of 4% human serum albumin were defined as 100% (CTL). (B) HepG2 cells were treated with 1 mM KCl or 1 mM IS for 24 h. (B1) Western blots of lysates isolated from HepG2 cells treated with KCl or IS were probed with fetuin-A or α-tubulin antibodies. (B2) Fetuin-A protein levels were quantified using α-tubulin as an endogenous reference; fetuin-A levels without KCl and IS were defined as 100% (CTL). (C) HepG2 cells were treated with 0.53 or 1.06 mM PCS or 1 mM IS for 24 h. (C1) Western blots of cell lysates isolated from HepG2 cells were probed with fetuin-A or α-tubulin antibodies. (B2) Fetuin-A protein levels were determined using α-tubulin as an endogenous reference, and fetuin-A levels without IS and PCS were defined as 100% (CTL). (D) HepG2 cells were treated with 1.06 mM PCS or 1 mM IS or with both 1.06 mM PCS and 1 mM IS for 24 h. (D1) Western blots of lysates isolated from HepG2 cells treated with 1.06 mM PCS or 1 mM IS or with both 1.06 mM PCS and 1 mM IS were probed with fetuin-A or α-tubulin antibodies. (D2) Fetuin-A protein levels were quantified using α-tubulin as an endogenous reference; fetuin-A levels without PCS and IS were defined as 100% (CTL). Means ± SDs were calculated from at least three independent experiments. *P < 0.05 versus CTL.
AhR was necessary for the interaction between IS and XRE, because recent findings suggested that AhR does not always interact with XRE during the ligand-activated expression of some genes [29]. IS-induced luciferase activity was suppressed in HepG2 cells transfected with siRNA specific to AhR (Figure 5A2). Therefore, these findings suggest that IS elicited
The involvement of AhR in IS-induced suppression of fetuin-A expression

Treating HepG2 cells with either 1 mM IS or 30 nM TCDD suppressed fetuin-A expression significantly (Figure 5B1 and B2), suggesting the involvement of AhR in this effect of IS. To confirm whether AhR is necessary for IS-suppressed fetuin-A expression, we knocked down AhR in HepG2 cells (Figure 6A1). Importantly, IS-induced fetuin-A suppression was restored in cells in which AhR had been knocked down (Figure 6A2). These findings suggest that IS could suppress hepatic fetuin-A expression via the AhR.

No effect of IS on fetuin-A expression via AhR in primary cultured hepatocytes

Finally, we examined the inhibitory effects of IS on fetuin-A expression in primary cultured hepatocytes. IS had no effects on fetuin-A expression in both mouse (Figure 7B1 and B2) and human primary hepatocytes (Figure 7C1 and C2). In contrast to HepG2 cells and human primary hepatocytes, little expression of AhR was detected in mouse primary hepatocytes (Figure 7A). Moreover, treatment with 0.5 and 1 mM IS for 24 h down-regulated AhR expression in human primary hepatocytes (Figure 7C1 and C3). Therefore, little and altered expression of AhR might result in unchanged levels of fetuin-A expression despite the presence of IS in primary hepatocytes.
DISCUSSION

In the current study, we demonstrated that IS, a representative uraemic toxin, suppressed fetuin-A expression directly in cultured hepatocytes. The IS-induced suppression of fetuin-A expression was independent of MAPK signalling pathways and ROS generation. However, the dioxin receptor AhR was involved in IS-suppressed fetuin-A expression.

Patients undergoing dialysis are susceptible to the effects of uraemic toxins, including IS [18, 19, 31]. A previous study showed that IS stimulated ROS production in human umbilical vein endothelial cells (HUVECs) [26]. IS-induced ROS enhanced the expression of the pro-inflammatory protein monocytic chemoattractant protein-1 (MCP-1) by activating the ERK1/2 and p38 pathways in HUVECs [28]. IS also activated ERK1/2 in vascular smooth muscle cells (VSMCs) to promote their proliferation [32]. Furthermore, IS-induced oxidative stress up-regulated the expression of osteoblast-specific proteins in VSMCs, which may be involved in vascular calcification [27]. IS directly stimulated cardiac myocyte hypertrophy and fibrosis via activation of ERK 1/2 and p38, which may explain the uraemia-associated comorbidity chronic heart failure [33]. Therefore, in the current study, we assessed the involvement of ROS and MAP kinase signalling pathways in IS-suppressed fetuin-A expression in cultured hepatocytes. However, our data suggest that IS-suppressed fetuin-A expression was not downstream of ROS generation and/or MAP kinase activation (Figures 3 and 4).

Emerging evidence suggests that IS can exert diverse toxic effects via activation of AhR [20–23]. AhR is a ligand-activated transcription factor that belongs to the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family [29, 30]. The binding of cognate ligands to AhR in the cytoplasm leads to its translocation into the nucleus, where it dimerises with AhR nuclear translocator (ARNT). The AhR : ARNT complex then transcribes target genes by binding to XREs or dioxin-responsive elements in the promoter region [29, 30]. Historically, the activation of AhR has been investigated extensively using environmental pollutants such as TCCD and polychlorinated biphenyls (PCBs) in the field of carcinogenesis. Both IS and TCCD suppressed fetuin-A expression (Figure 5), suggesting that IS may be an endogenous ligand for AhR, particularly under uraemic conditions.
However, IS-activated AhR did not seem to suppress fetuin-A expression in a direct fashion, because there is no putative XRE within the fetuin-A promoter [34]. A strong basal fetuin-A transcription was maintained by long isoforms of CCAAT enhancer-binding protein (C/EBP) possessing complete transactivation domain. Whereas pro-inflammatory cytokines induced replacement of long C/EBP isoforms by short C/EBP isoforms lacking of the transactivation domain, resulting in suppression of fetuin-A [35]. In contrast, it has been shown that C/EBP β and hepatocyte nuclear factor 3β (HNF 3β) were involved in dexamethasone-induced up-regulation of fetuin-A [36]. Moreover, it has been reported that fatty acids could increase fetuin-A expression through enhancement of nuclear factor κB (NF-κB) binding to the fetuin-A promoter directly [37]. Except classical AhR : ARNT pathway, components of NF-κB can form heterodimers with AhR, leading to diverse biological effects [29]. Therefore, further studies are required to investigate the interactions and crosstalk between the AhR and various transcriptional factors including C/EBPs, HNF 3β, and NF-κB in IS-induced fetuin-A suppression.

The present study has several limitations. First, we mainly used human hepatoma HepG2 cells. Unexpectedly, IS had no effects on fetuin-A expression in mouse and human primary cultured hepatocytes, possibly due to different reasons. In mouse primary hepatocytes, little expression of AhR was detected compared with that in human hepatocytes (Figure 7A). Thus, IS could not exert considerable inhibitory effects on fetuin-A via AhR. In addition, Schroeder et al. reported that IS demonstrated a 500-fold greater potency with regard to transcriptional activation of the human AhR relative to the mouse AhR in the hepatoma cell line [20]. Taken together, it is possible that the pathological role of IS may be different among humans and rodents. On the other hand, the expression of AhR in human primary hepatocytes was similar to that in HepG2 cells (Figure 7A). However, IS clearly suppressed AhR expression only in human primary hepatocytes using unknown mechanisms (Figure 7C1 and C3). This might lead to no change of fetuin-A expression in total. Another possibility may be that basal fetuin-A expression in human primary hepatocytes was much lower than that in HepG2 cells. Thus, it could be difficult to detect significant effect of IS on fetuin-A suppression in human primary hepatocytes. We also have to consider the origin and inherent characteristics of primary hepatocytes. Further studies will be required to clarify these issues using other lines of human primary hepatocytes.

Second, in addition to in vitro findings, in vivo and/or clinical studies will be necessary to confirm whether IS could suppress fetuin-A expression in the liver. Although we will possibly utilize the CKD animal model, such as sub-totally nephrectomized mice or rats, the species difference should be considered, that is little expression of AhR and low activation of IS-induced AhR in mouse hepatocytes. For example, a recent report showed that IS caused podocyte injury and glomerular damage through activation of AhR in podocytes [38]. In the present study, an ~4-fold concentration of IS was required to injure mouse podocytes (cell viability) compared with human podocytes. To clarify the significance of IS in uremic patients, it will be interesting to examine the association of serum IS levels with serum fetuin-A levels in clinical studies. Though we theoretically expect an inverse relationship between IS and fetuin-A, the presence of various other factors including uremic toxins also have to be considered. Recently, indole-3 acetic acid (IAA), one of the indole derivatives, as well as IS, was a significant predictor of mortality and cardiovascular events in patients with CKD [39]. IAA also activated AhR in HUVECs [39], although IAA was relatively weaker than IS in AhR activation [20]. Thus, we might have to pay attention to the combination effects of uremic toxins on fetuin-A expression in uremic patients.

Finally, because IS is one of the protein-bound toxins, the following two points should be considered: (i) the concentrations of IS and (ii) the presence of albumin in experimental studies [40]. In the present study, 1 mM (=212 mg/L) of IS was mainly used. According to this review [40], the highest individual value of IS is 236.0 mg/L in uraemia. Therefore, a concentration of 1 mM of IS is possible in humans. Likewise, the highest mean of IS is 44.5 mg/L which may occur more frequently in uremic patients. This concentration is close to 0.25 mM (=53 mg/L). In the present study, 0.25 mM IS significantly suppressed both the mRNA and protein expressions of fetuin-A (Figure 1). Vanholder et al. [40] suggested that the presence of 40 g/L albumin seems to be ideal in in vitro studies (40 g/L = 4 g/dL = 4% albumin). As shown in Figure 2, the effect of 0.1 mM IS on fetuin-A suppression was not altered in the presence of 4% albumin. Vanholder et al. [40] described that a high concentration was applied essentially in vitro, where the concentration should be considered to compensate for short exposure to uremic toxins, in contrast to real life. Although the optimal condition has not been determined, extremely high free concentrations of IS and inappropriately low albumin should be avoided in future studies.

In conclusion, the present study demonstrated that IS suppresses fetuin-A expression directly via an AhR-dependent pathway in hepatocytes. This finding suggests that IS could affect fetuin-A deficiency-mediated cardiovascular damage in addition to its direct toxic effects on the vascular wall. This may be one mechanism underlying the high cardiovascular mortality of dialysis patients with low fetuin-A levels. These findings also provide insight into the clinically important relationship between fetuin-A deficiency and the high cardiovascular mortality in dialysis patients.

**AUTHORS’ CONTRIBUTIONS**

A.O. and S.N. generated the data. K.Mor conceived the research hypothesis and wrote the manuscript. M.E., T.M., K.Mot, S.F., Y.I., T.S., E.I. and M.I. contributed to the discussion and reviewed and edited the manuscript. K.Mor is the guarantor of this work, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**ACKNOWLEDGEMENTS**

We thank Masayo Monden for her technical assistance. We also thank Dr Hideki Uedono for additional experiments. This
CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

33. Lekavanijit S, Adrahtas A, Kelly DJ et al. Does indoxyl sulfate, a uremic toxin, have direct effects on cardiac fibroblasts and myocytes? Eur Heart J 2010; 31: 1771–1779