Indoxyl Sulfate Upregulates Liver P-Glycoprotein Expression and Activity through Aryl Hydrocarbon Receptor Signaling

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ABSTRACT

In patients with CKD, not only renal but also, nonrenal clearance of drugs is altered. Uremic toxins could modify the expression and/or activity of drug transporters in the liver. We tested whether the uremic toxin indoxyl sulfate (IS), an endogenous ligand of the transcription factor aryl hydrocarbon receptor, could change the expression of the following liver transporters involved in drug clearance: *SLC10A1*, *SLC22A1*, *SLC22A7*, *SLC47A1*, *SLCO1B1*, *SLCO1B3*, *SLCO2B1*, *ABCB1*, *ABCB11*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC6*, and *ABCG2*. We showed that IS increases the expression and activity of the efflux transporter P-glycoprotein (P-gp) encoded by *ABCB1* in human hepatoma cells (HepG2) without modifying the expression of the other transporters. This effect depended on the aryl hydrocarbon receptor pathway. Presence of human albumin at physiologic concentration in the culture medium did not abolish the effect of IS. In two mouse models of CKD, the decline in renal function associated with the accumulation of IS in serum and the specific upregulation of *Abcb1a* in the liver. Additionally, among 109 heart or kidney transplant recipients with CKD, those with higher serum levels of IS needed higher doses of cyclosporin, a P-gp substrate, to obtain the cyclosporin target blood concentration. This need associated with serum levels of IS independent of renal function. These findings suggest that increased activity of P-gp could be responsible for increased hepatic cyclosporin clearance. Altogether, these results suggest that uremic toxins, such as IS, through effects on drug transporters, may modify the nonrenal clearance of drugs in patients with CKD.

Patients with CKD often require the prescription of numerous medications related to the disease and its complications and comorbidities.[1,2] Changes in drug disposition make dose adjustments challenging in these patients, with frequently occurring drug-related adverse events.[3,4]

CKD not only alters the elimination of drugs excreted by the kidneys, but it also affects the metabolism of drugs subject to nonrenal clearance, which involves mainly the liver and intestines.[5,6] Biotransformation

and transport across cellular membranes are two essential cellular processes that control drug tissue distribution from the entry in the body to its elimination.[7,8] How an uremic environment could modify these two processes is poorly understood[5] due to the multiplicity and interplay of processes leading to the drug distribution and the broad substrate specificity of metabolic enzymes and transporters. *In vitro* studies using hepatocytes cultured with human uremic serum and animal models of CKD showed a reduced expression and direct inhibition of cytochrome P450 enzymes.[6,9–11] A modification of expression and/or activity of some drug transporters, such as OATP1B1, OATP1B3, MRP4, BCRP, and P-glycoprotein (P-gp), is also observed in the context of uremia.[6,12–15] These changes could be attributed to circulating molecules associated with CKD, such as uremic toxins.

Uremic solutes or toxins are compounds normally eliminated by the kidneys but accumulated in the blood and tissues of patients with CKD (http://www.uremic-toxins.org).[16] They display a large variety in chemical structures and are classified into three groups according to their size and dialysis clearance: the small water-soluble compounds (molecular mass <500 D), the middle molecules (molecular mass >500 D), and the protein-bound compounds.[16] The indolic uremic toxin indoxyl sulfate (IS), an end product of tryptophan metabolism by gut microbiota, is a protein-bound toxin poorly removed by dialysis.[16] IS is a nephrovascular toxin, and its levels correlate with mortality in patients with CKD.[17–19] It displays deleterious effects on endothelial and smooth vascular muscle cells that might participate in the adverse cardiovascular outcomes in patients with CKD.[20] IS may also accelerate CKD progression; it induces fibrosis in rat kidneys *via* an increased expression of profibrotic and proinflammatory genes in tubular cells.[21] The cellular receptor for IS is the ubiquitous transcription factor aryl hydrocarbon receptor (AhR), well known to mediate the toxicity of the widespread environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).[22–24] We have shown that the activation of AhR by IS in primary endothelial cells (human umbilical vein endothelial cells) leads to the increased expression of a part of the prototypic pattern of AhR-induced genes, such as those involved in detoxification processes.[24]

The aim of this study was to determine if IS could modify the expression of transporters known to be involved in the hepatic clearance of drugs and whether the AhR pathway is involved in this process. We first tested this hypothesis *in vitro* in the hepatocarcinoma cell line HepG2. We then investigated the effect of CKD on the hepatic expression of P-gp using two mouse models of CKD (adenine diet and remnant kidney models). Finally, we highlight the clinical relevance of our observations in a cohort of patients with CKD.

RESULTS

IS Upregulated the Gene Expression of *ABCB1* in HepG2 Cells

We tested whether IS altered the expression of genes encoding the drug liver transporters listed in Table 1.[25–27] HepG2 cells were incubated for 16 hours with IS at the uremic concentration of

Significance Statement

CKD changes the pharmacokinetics of many drugs, both those with renal and nonrenal clearance, leading to reduced effectiveness and an increase in secondary effects. This paper describes effects of indoxyl sulfate, a uremic toxin, on hepatocyte expression and activity of P-glycoprotein operating through an aryl hydrocarbon receptor pathway. Studies show that P-glycoprotein expression is increased in uremic mice. In transplant recipients with CKD, high levels of indoxyl sulfate are associated with increases in the required dose of cyclosporin, a substrate of P-glycoprotein. Thus, indoxyl sulfate increases P-glycoprotein expression and can modify the pharmacokinetics of drugs. Monitoring indoxyl sulfate levels could improve drug management in patients with CKD.

0.2 mM.[28] Because IS is synthesized as a potassium salt, cells were also incubated with control medium containing KCl at 0.2 mM. IS only increased the expression of *ABCB1*, whereas no effect was observed on the other transporters screened (Table 1). *ABCB1* gene encodes the transporter P-gp, which is also known as multidrug resistance gene 1. Dose-response experiments showed that IS increased the *ABCB1* mRNA levels at 0.2, 0.4, and 1 mM by 81%, 120%, and 176%, respectively (Figure 1A). *ABCB1* gene was overexpressed after 16 hours of incubation with IS and remained high until 48 hours (Figure 1B).

IS Increased the Protein Expression and Activity of P-gp

We tested whether IS increased the amount and activity of P-gp in HepG2 cells after 16 hours of incubation. Flow cytometry analysis revealed that IS at 0.2, 0.4, and 1 mM significantly increased the amounts of P-gp by 22%, 52%, and 51%, respectively (Figure 2A). By Western blot analysis, IS at 0.2 and 1 mM increased the amount of P-gp by 1.7- and 2.5-fold, respectively (Figure 2B). The efflux activity of P-gp was evaluated using its fluorescent substrate rhodamine 123 (R123). Flow cytometry analysis showed that IS at 0.08, 0.2, 0.4, and 1 mM decreased the intracellular concentrations of R123 by 13%, 19%, 34%, and 44%, respectively (Figure 2C), whereas at 0.03 and 0.01 mM, IS did not have an effect. The increase in P-gp activity was inhibited by P-gp inhibitors elacridar at 2 μM and verapamil at 50 μM (Supplemental Figure 1).

To confirm the effects observed on tumoral cell line, we used human primary hepatocytes from three donors to study the effect of IS on the R123 efflux. These primary cells are more sensitive to IS than HepG2, because mortality is high in the presence of IS at 1 mM. The responses of the donors displayed heterogeneity: one sample showed no response, the second showed a weak effect (18% of reduction at 0.2 mM IS), and the third showed a higher effect (36% of reduction with 0.2 mM IS) (Figure 2D).

Human Albumin Did Not Alter the Effects of IS during Short and Long Incubations

In blood, albumin binds to IS.[29] Because culture medium contains only 2.5 g/L of albumin, we added human albumin at 40 g/L (Figure 3). Physiologic concentration of albumin did not affect the effects of IS on ABCB1 mRNA levels and P-gp activity after 16 hours of incubation (Figure 3, A and B).

Table 1. Expression of transporter genes after incubation of HepG2 cells with IS

Gene (Transporter)	Drug Substrates[25–27]	Fold Change of Expression after Incubation with IS
SLC10A1 (NTCP)	Atorvastatin, fluvastatin, pitavastatin, rosuvastatin	No change
SLC22A1 (OCT1)	Aciclovir, berberine, cimetidine, *cis*-diammine(pyridine) chloroplatin (ii), furamidine, ganciclovir, irinotecan, lamuvidine, metformin, oxaliplatin, palitacel, pentamidine, picoplatin, quinine, quinidine, zidovudine	No change
SLC22A7 (OAT2)	5-Fluorouracil, allopurinol, bumetanide, cimetidine, erythromycin, methothrexate, paclitaxel, ranidine, salicylate, taxol, theophylline, tetracyclin, zidovudine	No change
SLC47A1 (MATE1)	Acyclovir, cephadrine, cephalexin, cimetidine, fexofenadine, gancyclovir, metformin, oxaliplatin, procainamide	No change
SLCO1B1 (OATP1B1)	Asunaprevir, bosentan, cerivastatin, danoprevir, enalapril, olmesartan, phalloidin, repaglinide, statins, temocaprilat, valsartan	No change
SLCO1B3 (OATP1B3)	Amanitin, bosentan, digoxin, docetaxel, enalapril, erythromycin, fexofenadine, fluvastatin, methotrexate, ouabain, paclitaxel, phaloidin, pitavastatin, rifampicin, rosuvastatin, statins, telmisartan, valsartan	No change
SLCO2B1 (OATP2B1)	Atorvastatin, benzylpenicillin, bosentan, ezetimibe-glucuronide, fexofenadine, fluvastatin, glibenclamide, montelukast, pitavastatin, pravastatin, rosuvastatin	No change
ABCB1 (MDR1; PGP)	Amiodarone, berberine, bisantrene, carbamazepine, celiprolol, chloroquine, colchicine, cyclosporin, daunorubicin, desipramine, digitoxin, digoxin, docetaxel, doxorubicin, erythromycin, etoposide, fexofenadine, grepafloxacin, imatinib, indinavir, ivermectin, levofloxacin, lidocaine, losartan, lovastatin, irinotecan, loperamide, methadone, methotrexate, mibefradil, mitoxantrone, morphine, nelfinavir, ortataxel, paclitaxel, ritonavir, saquinavir, seliciclib, sirolimus, sparfloxacin, sumatriptan, tacrolimus, talinolol, terfenadine, topotecan, vecuronium, vinblastine, vincristine	2.5 ± 0.2
ABCB11 (BSEP)	5-Fluorouracil, pravastatin, vinblastine	No change
ABCC2 (MRP2)	Cisplatin, doxorubicin, etoposide, glutathione and glucuronide conjugates, glucuronidated SN-38, methotrexate, mitoxantrone, olmesartan, valsartan, vinblastine	No change
ABCC3 (MRP3)	Acetaminophen glucuronide, etoposide, fexofenadine, methotrexate, teniposide, vincristine	No change
ABCC4 (MRP4)	Methotrexate	No change
ABCC6 (MRP6)	Etoposide	No change
ABCG2 (BCRP)	Abacavir, albendazole sulfoxide, anthracyclines, camptothecin, cerivastatin, cimetidine, ciprofloxacin, daunorubicin, dipyridamole, doxorubicin, edaravone, erlotinib, flavopiridol, gefitinib, glibenclamide, imatinib, irinotecan, lamivudine, methotrexate, mitoxantrone, nelfinavir, nitrofurantoin, norfloxacin, nucleoside analogs, ofloxacin, olmesartan, oxfendazole, pantoprazole, pitavastatin, prazosin, rosuvastatin, SN-38, statins, sulfasalazine, topotecan, zidovudine	No change

Representative substrates for drug transporters in HepG2 cells and their expression evaluated by RT-PCR after exposure to IS (0.2 mM) for 16 hours. Analyses used a cutoff of twofold upregulated genes compared with control cells (KCl; 0.2 mM). Data are expressed as mean$\pm$SEM of four independent experiments. MDR1, multidrug resistance gene 1.

To mimic *in vivo* conditions (long-term exposure with lower concentrations of IS), HepG2 was incubated with lower concentrations of IS (0.08, 0.03, and 0.01 mM) for 96 hours instead of 16 hours with or without human albumin. IS at 0.03 and 0.08 mM increased both *ABCB1* mRNA level (by 72% and 98%, respectively) and P-gp activity (intracellular concentration of R123 was decreased by 17% and 21%, respectively) (Figure 3, C and E). IS at 0.01 mM increased mRNA levels of P-gp but did not increase its activity. The same results were obtained in the presence of human albumin (Figure 3, D and F).

AhR Pathway Is Involved in the Induction of P-gp by IS
Because IS is an endogenous ligand of the transcription factor AhR, we studied the involvement of AhR pathway in the effect of IS on P-gp expression by using small interfering RNA (siRNA) targeted against AhR.[24] AhR siRNA induced a

significant decrease in *AhR* mRNA and protein levels (Supplemental Figure 2). The increased expression of P-gp induced by IS was completely abolished by AhR silencing at mRNA and protein levels, whereas scrambled control siRNA had no effect (Figure 4, A and B). Consequently, in cells treated with AhR siRNA, IS did not lead to an increased efflux activity of P-gp (Figure 4C). Furthermore, TCDD, the most well known agonist of AhR, increased gene expression of *ABCB1*, P-gp level (2.4-fold increase), and its efflux activity (Figures 4, D–F).

P-gp Expression Is Increased in Two Mice Models of CKD
The adenine diet[30] and the remnant kidney (5/6 nephrectomy)[31] models of CKD were used to test whether the uremic environment could modify the liver expression of the *ABCB1* mouse ortholog genes *Abcb1a* and *Abcb1b*. The two mice models of CKD showed significant increased plasma concentrations of creatinine, blood
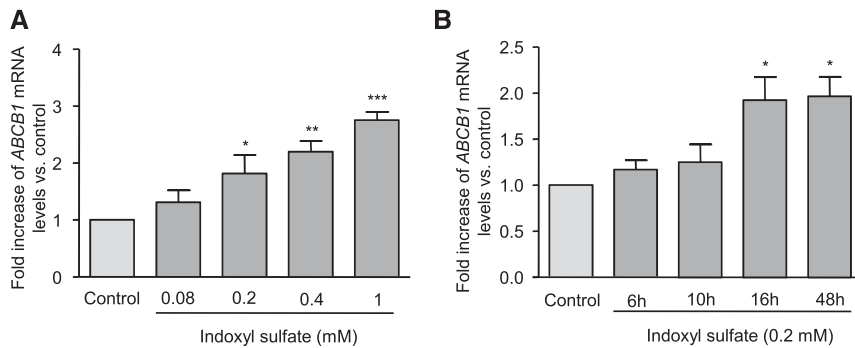
Figure 1. IS increased the expression of *ABCB1* in HepG2 cells. HepG2 cells were incubated with IS at (A) different concentrations and (B) different incubation times. mRNA levels of ABCB1 were quantified by quantitative comparative RT-PCR. Data are expressed as mean±SEM of (A) four and (B) six independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

urea nitrogen, and IS (Figure 5, A–C), with a greater increase in the adenine diet model compared with the 5/6 nephrectomy model. In the adenine diet model, the kidney disease is induced by the deposition of adenine crystals in the kidneys, and we investigated whether liver could suffer from such damages. Histologic sections showed the presence of crystals in kidneys but not in livers (Supplemental Figure 3) as previously described.[32] An increase in mRNA levels of *Abcb1a* was detected in the liver of mice with CKD compared with control mice, with an effect being more pronounced in adenine diet (Figure 5D). *Abcb1b* was only increased in 5/6-nephrectomized mice (Figure 5E).

Patients with Higher Levels of IS Needed to Take Higher Doses of Cyclosporin

We studied 109 patients with CKD (eGFR<60 ml/min per 1.73 m²; stages 3–5) under immunosuppressive treatment with cyclosporin after kidney or heart transplantation. Baseline characteristics of patients are listed in Table 2. Patients exhibited significantly increased levels of indolic uremic toxins (median [25th–75th percentile]; IS: 4.2 μM [0.06–11.7]; indole-3-acetic acid [IAA]: 1.9 μM [1.3–2.7]). Because of the large pharmacokinetic variability, treatment with cyclosporin needs obligatory therapeutic drug monitoring. To achieve the efficient blood target concentration, the correct dose of cyclosporin is then adjusted for each patient. Multivariate analysis of parameters that influence the dosage of cyclosporin showed that cyclosporin dosage requirement was significantly associated with levels of IS ($P<0.05$) (Table 3). We did not find a correlation between IAA levels and this ratio. Patients with higher levels of IS were observed to require higher doses of cyclosporin to reach the efficient blood target level needed to prevent transplant rejection.

DISCUSSION

We showed that IS increases the expression and activity of P-gp in HepG2 cells in an AhR-dependent way. P-gp is an efflux

pump exporting lipophilic substrates, including multiple drugs. It is expressed mainly in epithelia with a barrier function (brain, liver, intestine, and kidneys).[33] It is involved in the decreased drug accumulation and development of resistance to various drugs. In mice models of CKD, the decline in renal function led to higher IS serum levels and an upregulation of liver *Abcb1a* expression. In patients with transplants and CKD treated by cyclosporin, which is a P-gp substrate, patients with higher serum levels of IS required higher doses of cyclosporin to achieve the serum target concentration.

A large gap exists between the observation of altered clearance of drugs in patients with CKD and the knowledge of mechanisms involved.[5] We, therefore, tested the effect of IS on the expression of the main transporters known to be involved in drug clearance by the liver.[6] We used the cell line HepG2 derived from a human hepatocellular carcinoma, because the expression of efflux transporters and cell polarity was maintained.[34–36] These characteristics make HepG2 an adequate tool to study transporters expression.

We found that, except for P-gp, IS did not affect the mRNA levels of the other drug transporters analyzed. However, we could not exclude the possibility that IS can affect their activity in another way, such as by competitive inhibition. Indeed, IS and two other uremic toxins, kynurenic acid and IAA, have been shown to directly inhibit SCLO1B1- and SCLO1B3-mediated transport[15] as well as MRP4 (*ABCC4*) and BCRP (*ABCG2*)–mediated efflux.[12] Tsujimoto *et al.*[37] showed that treatment of a hepatocellular carcinoma cell line (Hep3B) with uremic serum resulted in downregulation of the expression of OATP1B1 and OATP2B1 transporters. We were not able to confirm these results, but we evaluated here only the effect of IS. In addition, in CKD rats, MRP6 protein levels were reduced in the liver and kidney tissues, whereas its mRNA levels (*ABCC6*) were unchanged, pointing to post-translational mechanisms.[38]

To our knowledge, our study is the first to show that IS increases P-gp expression and its activity in HepG2 cells. We used a large range of IS concentrations (0.01–1 mM), because patients with CKD exhibit variable levels of IS (mean uremic concentration, 0.108 mM; highest value reported, 0.209 mM). Because in our cohort of patients with transplants, renal function is partly retained (39.3 ml/min per 1.73 m²), the mean concentration (0.010 mM) and the highest value of IS (0.126 mM) are lower. We observed an effect on *ABCB1* mRNA level and P-gp for the highest concentrations of IS used (1, 0.4, and 0.2 mM). At 0.08 mM, a slight increase was observed, which was significant only for the activity of P-gp and was not significant for *ABCB1* mRNA and P-gp levels. At 0.03 and 0.01 mM, IS did not affect P-gp. Thus, we ask if IS could act when used at these lower concentrations but after a longer
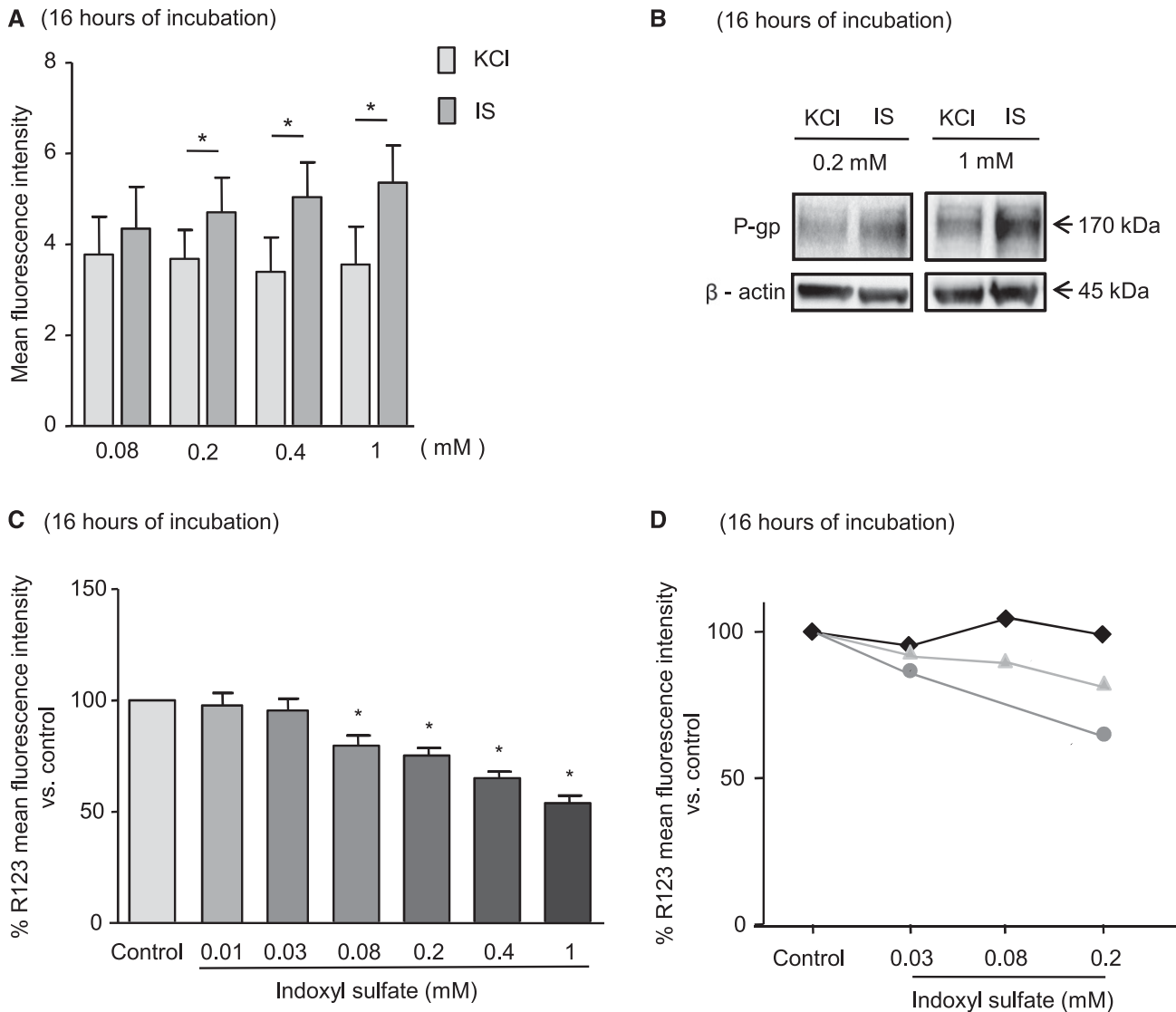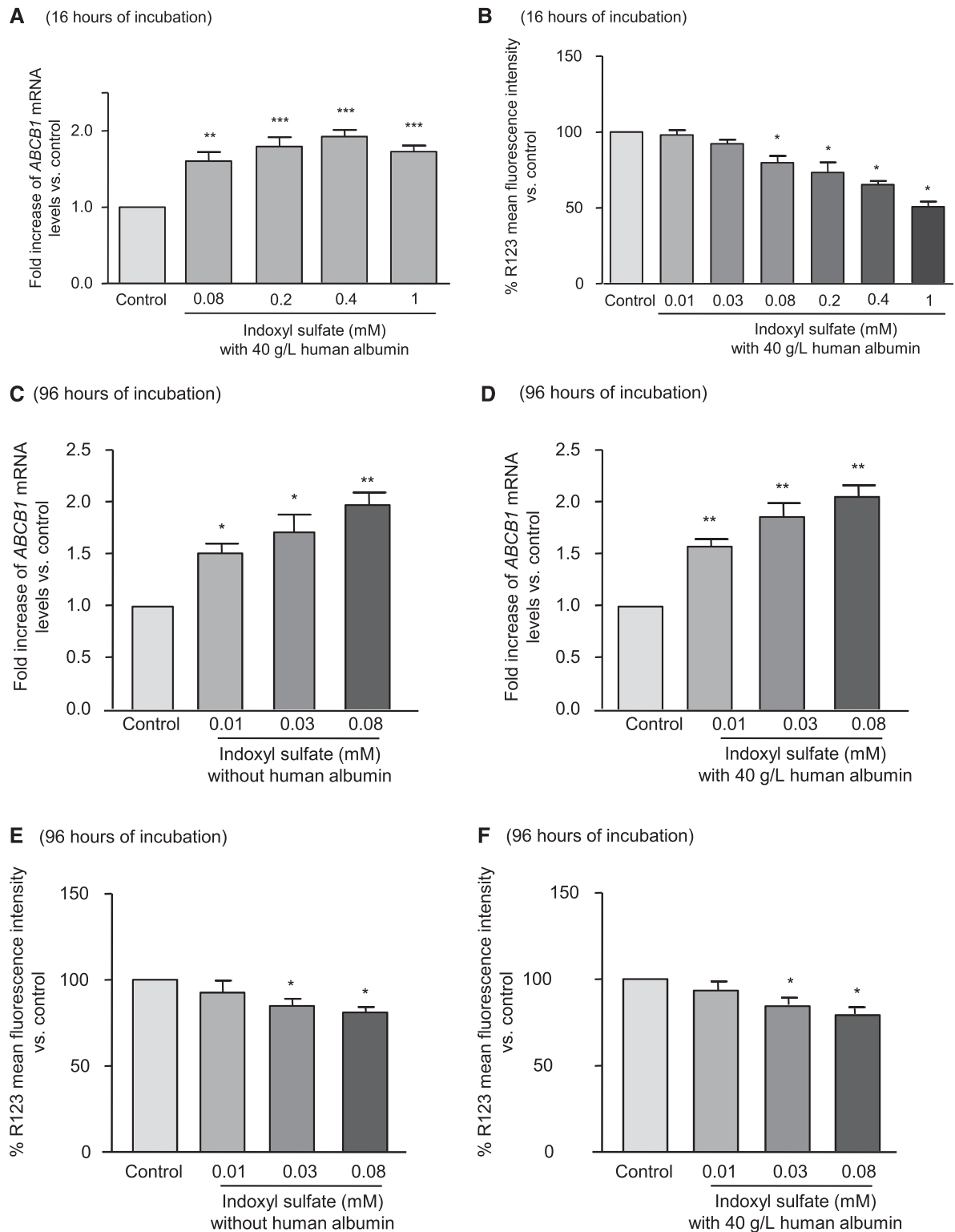
Figure 2. IS increased the expression and activity of P-gp in HepG2 cells and the activity of P-gp in human primary hepatocytes. The expression of P-gp was evaluated in HepG2 by (A) flow cytometry and (B) Western blot. (C and D) The transporter efflux activity was evaluated by using its fluorescent substrate R123 (5 μM) in (C) HepG2 or (D) three samples of human primary hepatocytes. (A and C) Data are expressed as mean±SEM of six independent experiments. *$P<0.05$.

time of incubation (96 hours instead of 16 hours) to mimic the *in vivo* conditions. After 96 hours of incubation at 0.08 and 0.03 mM, IS increased *ABCB1* mRNA and P-gp activity. At 0.01 mM, no effect of IS was observed. The values of IS for which we observed an effect were in the range of those of our patients. Incubation for several days with concentrations found in patients certainly represents a more realistic situation than shorter incubations with supraphysiologic concentrations. Indeed, patients with CKD are continuously exposed systematically to uremic toxins, especially protein-bound compounds, which are badly removed by dialysis. In addition, we tested the effect of IS on three samples of human primary hepatocytes. The results obtained for the efflux of R123 display heterogeneity. Jigorel *et al.*[39] found similar responses in terms

of P-gp induction by TCDD, which extended from no response to threefold induction. Indeed, the high functional variability of these primary cells is well known and constitutes the obstacle for their use in routine.[34] Despite this problem, we found that IS increases the efflux of R123 in human primary hepatocytes, because two of the three different samples tested have responded to it, suggesting that this effect of IS could take place *in vivo*.

This increase in P-gp expression induced by IS was AhR dependent, because AhR silencing abolished these effects. Indolic uremic toxins, such as IS, IAA, and indole-3-carbinol, are known to activate AhR.[22,40,41] Schroeder *et al.*[22] showed that IS activates AhR by direct binding. We have previously shown that IS and IAA increase cyclo-oxygenase 2 and tissue factor in

A (16 hours of incubation)

B (16 hours of incubation)

C (96 hours of incubation)

D (96 hours of incubation)

E (96 hours of incubation)

F (96 hours of incubation)

Figure 3. Human albumin did not modify the effects of IS during short or long incubations. Analysis of ABCB1 mRNA and P-gp activity in HepG2 cells incubated with IS or KCl (A and B) during 16 hours in the presence of human albumin or (C–F) for 96 hours with or without albumin. Results represent the mean±SEM of five independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

an AhR-dependent way.[24,42] P-gp is regulated by numerous transcription factors, among which are the xenosensor receptor pregnane X receptor, the constitutive androstane receptor, and the AhR.[40,43] AhR is a transcription factor mainly located in the cytoplasm and associated with Hsp90, XAP2, and p23.[44–46] Ligand binding to AhR leads to its activation and
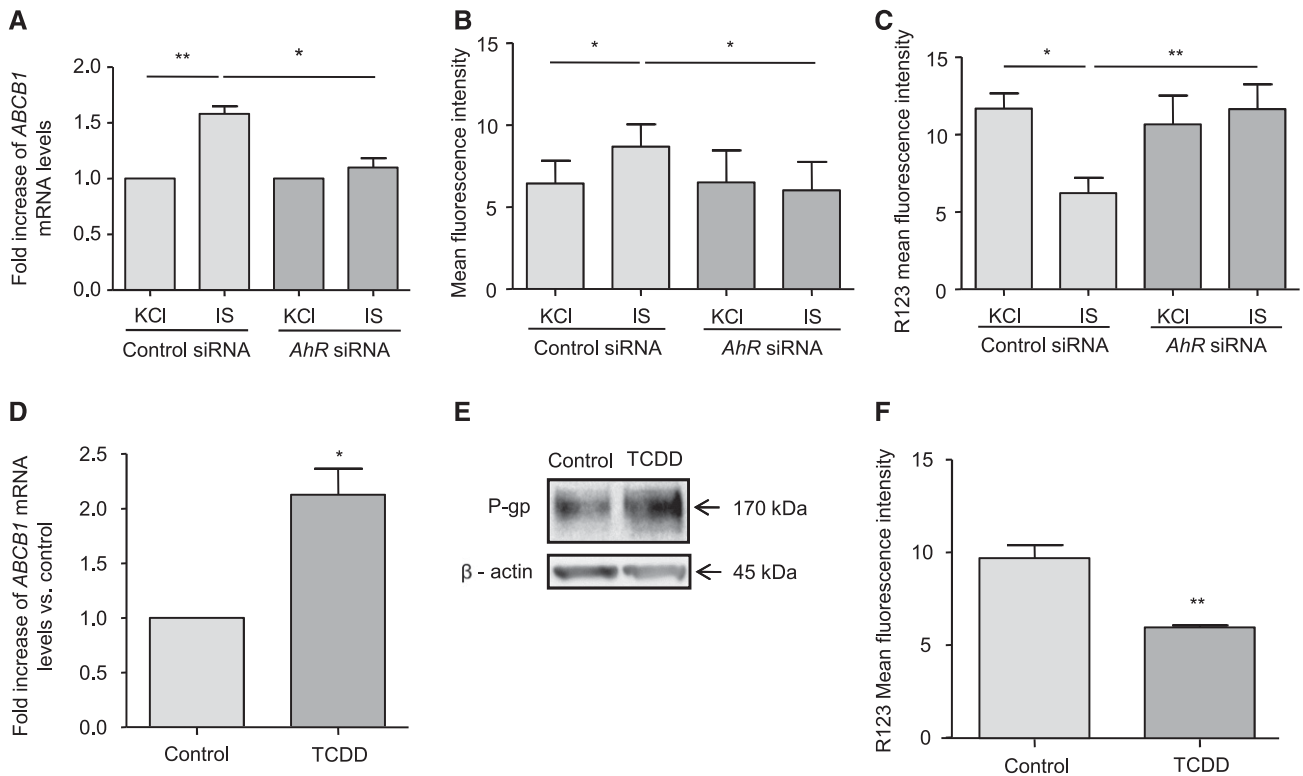
Figure 4. Induction of P-gp expression and its activity by IS is dependent on the AhR pathway. (A–C) Analysis of ABCB1 mRNA, P-gp expression, and activity in HepG2 cells transfected with control siRNA or against *AhR* (*AhR* siRNA). (D–F) Analysis of *ABCB1* mRNA, P-gp expression, and P-gp activity in HepG2 cells incubated with TCDD (30 nmol/L). Results represent the mean±SEM of (A) five and (B–D and F) four independent experiments. *$P < 0.05$; **$P < 0.01$.

dissociation from these other partners. Then, AhR translocates to the nucleus, where in association with ARNT, it binds to specific DNA consensus sequences (the xenobiotic response element) in the promoters of target genes and induces their transcription.[44,45] In addition to this genomic pathway, AhR has been shown to act as a signaling molecule without DNA binding.[46,47] The involvement of AhR in the regulation of P-gp was not specific for IS, because TCDD has been shown to increase P-gp expression and activity in rat brain capillaries and reduce the accumulation of its substrates.[48] We also showed that TCDD was able to increase the expression and activity of P-gp in HepG2. In human hepatocyte primary culture, the response to TCDD in terms of P-gp expression is highly variable, ranging from no modification to an increase.[39] These results underline the complexity of P-gp regulation and could be explained by the facts that P-gp expression is under the control of multiple transcription factors and that each of them, like AhR, is involved in crosstalking with multiple signaling pathways.

We have shown that the effects of IS on P-gp expression are AhR dependent; however, the exact mechanism by which this is exerted is not elucidated. The increase in *ABCB1* mRNA level was significantly detectable after 16 hours of incubation with IS, suggesting that direct binding of AhR to the promoter region of *ABCB1* is not required. This is in accordance with the fact that xenobiotic response element was not found in its promoter region.[49] Therefore, P-gp overexpression due to AhR activation must be related to the nongenomic pathway.

IS belongs to protein-bound uremic toxins. The degree of protein binding is high, because the free fraction represents 10% of total amount of IS.[29] *In vivo*, albumin is the main binding protein for IS. The lack of albumin in culture medium can lead to an increase in the free fraction of IS. Thus, to approach the *in vivo* conditions of protein binding, we added to the culture medium: human albumin at 40 g/L. The presence of human albumin did not alter the effects of IS, suggesting that cellular uptake is not hampered by protein binding, which was recently shown for kidney proximal tubules[50] as well, and that the overall amount of IS, the free and the bound states, is required to act on AhR and P-gp.

In rodents, P-gp exists as two isoforms displaying 90% of homologies that are encoded by two closely located genes: Abcb1a and Abcb1b.[51] These isoforms are considered to fulfill the same function as the *ABCB1* gene in humans.[51] In the liver of two mice models of CKD, we found a significant increase in the mRNA expression of Abcb1a, whereas *Abcb1b* was only increased in 5/6-nephrectomized mice. The significant greater increase in mRNA level of Abcb1a in the adenine diet model could be related to the more advanced level of kidney disease in this model as suggested by the higher values of creatinine,
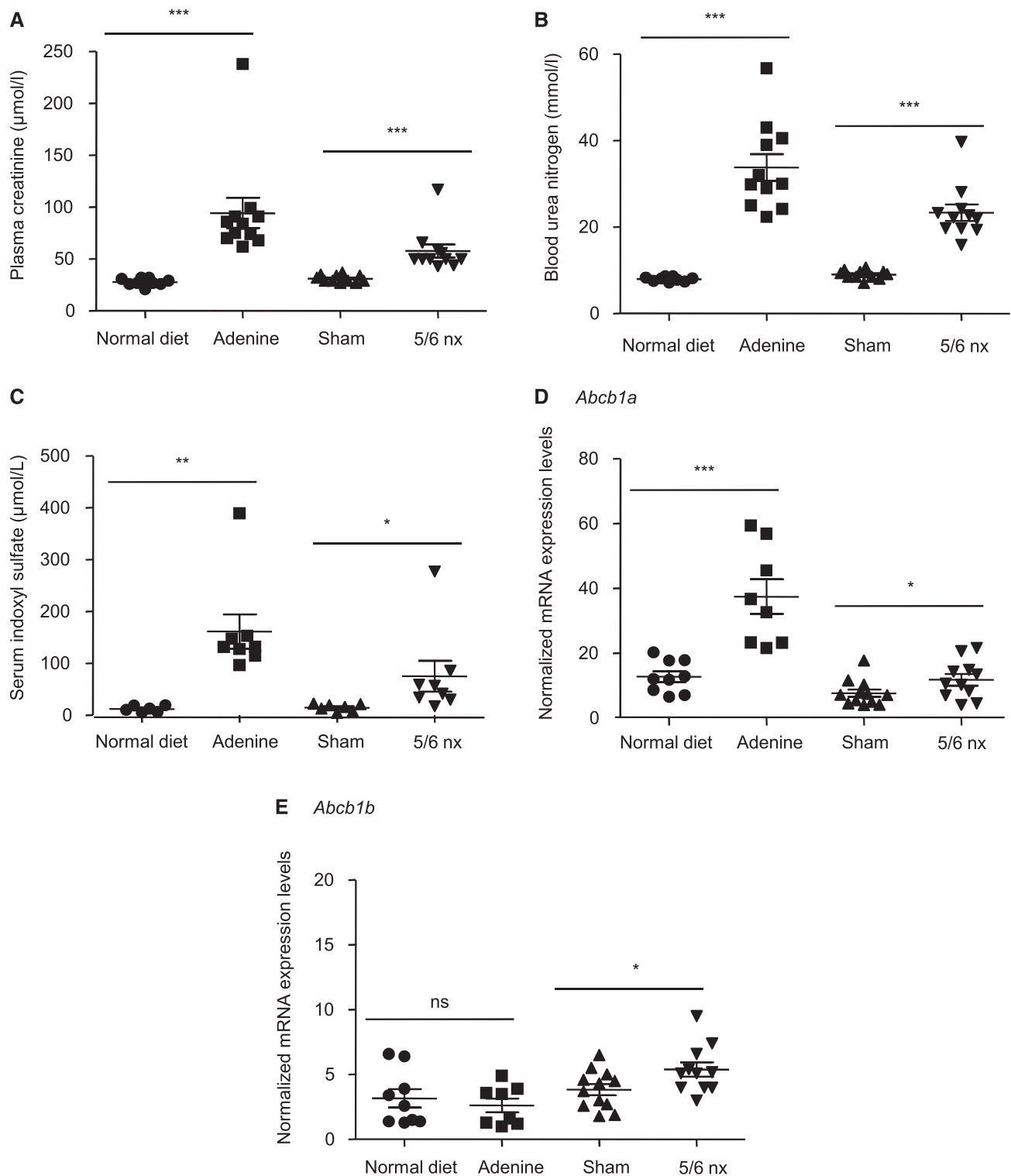
Figure 5. IS levels and *abcb1* expression are increased in two mice models of CKD. Kidney function in mice was assessed using (A) plasma creatinine and (B) BUN, which indicates a reduction in GFR in adenine-treated mice and 5/6-nephrectomized (5/6 nx) mice. (C) Serum concentrations of IS in mice were increased in the two models of CKD. (D and E) mRNA expression levels of two genes, *abcb1a* and *abcb1b*, that encode to P-gp in mice. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

urea, and consequently, IS. In addition, the fact that the *Abcb1b* mRNA level was only increased in nephrectomized mice suggested that metabolic modifications induced by

adenine diet prevent this effect. Our results in CKD mice are in accordance with previous work showing an increase in P-gp expression and activity in the liver of 5/6-nephrectomized

Table 2. Characteristics of heart and kidney transplant recipients with CKD

Transplant Recipients Characteristics	Total, *n*=109
Cyclosporin dosage, mg/d per 1 kg	1.81 (1.51–2.13)
Sex ratio, women/men	37/72
Age, yr	54.5 (47–62)
Body mass index, kg/m^2	22.78 (20.5–25.9)
Cyclosporin blood concentration, mg/L	87 (68.5–122.5)
Years after transplantation	6 (4–8)
eGFR (CKD-EPI), ml/min per 1.73 m^2	39.3 (32.2–50.1)
IS, μM	4.2 (0.06–11.7)
IAA, μM	1.9 (1.3–2.7)

Results from recipient characteristics represent the medians and the interquartile ranges (25th–75th percentiles). CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration.

rats.[52] Altogether, these results suggest that CKD is responsible for the increased expression of P-gp rather than the methods used to induce uremia (5/6 nephrectomy and adenine diet). In addition, because uremic sera from CKD rats[52] and patients with CKD[11] also led to an increase in P-gp activity in rat hepatocyte cell cultures, the authors suggested that seric factors may be involved. Because nothing was proven for other indole-derived compounds, we propose that uremic toxins, such as IS, could be some of the factors contributing to the upregulation of P-gp expression in CKD rodents.

In patients, we used an indirect method to analyze the activity of P-gp. The lipophilic nature of cyclosporin allows its entry into the cell by passive diffusion, but the exit of cyclosporin is dependent on the efflux transporter P-gp. Cyclosporin undergoes predominantly biliary clearance, with only 6% of renal excretion of unmodified cyclosporin.[53] In this context, coupling of P-gp and cyclosporin is interesting to study in patients, because P-gp seems to be a determinant for cyclosporin bioavailability. In patients with transplants and CKD on cyclosporin treatment, we observed a correlation between the serum levels of IS and the need for higher dosage of the

Table 3. Multiple linear regression modeling the relationship between cyclosporin dosage and IS levels

Cyclosporin Dosage, mg/d per 1 kg	Coefficient	95% CI	*P* Value
IS, μM	0.01	0 to 0.03	0.03
IAA, μM	−0.04	0.14 to 0.07	0.48
Cyclosporin target blood concentration, mg/L	0.010	0.006 to 0.015	<0.001
Recipient sex	0.44	0.06 to 0.82	0.02
Body mass index, kg/m^2	−0.09	−0.14 to −0.05	<0.001
Age, yr	−0.01	−0.03 to 0.01	0.18
Time since transplant	0	−0.05 to 0.04	0.83
eGFR (CKD-EPI), ml/min per 1.73 m^2	0.01	−0.01 to 0.02	0.33

R^2 value of the model: 0.54. CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration.

drug to achieve the target blood levels of cyclosporin. In other words, patients with higher levels of IS must take higher doses of cyclosporin to reach therapeutic levels to prevent transplant rejection. IS has been shown to slightly inhibit CYP3A4 at concentrations found in patients with CKD.[54,55] However, this should lead to an increase in cyclosporin bioavailability, which was not observed. Together, these findings support our results and lead to the hypothesis that CKD conditions could cause an upregulation of the efflux transporter P-gp in liver. This upregulation could be responsible for increased liver clearance of cyclosporin and a decrease in its bioavailability. In accordance with our results, a recent meta-analysis revealed that one of the most widely described polymorphisms responsible for an increase in P-gp activity, C3435T, was associated with higher dose requirements in kidney transplant recipients.[56,57] In addition, mice invalited for the *Abcb1a* gene, cannot eliminate cyclosporine and therefore display higher serum levels of this drug.[58] The absence of correlation between IAA and dose of cyclosporin suggests that IAA cannot modify P-gp expression in an AhR-dependent way. This result seems, at first glance, unexpected, because these two toxins exhibit a similar structure and both are able to activate AhR; however, it can be explained in several ways. IAA has a carboxyl group attached to the indole ring, and IS has a sulfate group. This difference could lead to specificity in the pattern of gene expression between them other than the classic pattern of AhR activation. According to this, IAA and IS display different binding degrees for AhR[22] that could be translated into differences in AhR activation and thus, differences in gene regulation. In addition, in clinical studies, where the levels of IS and IAA were measured simultaneously, common[24,59] but also specific[60–62] associations were found between these toxins and clinical parameters, arguing that not all indolic solutes act similarly. Finally, we could not exclude that IAA levels, which were lower than those of IS in our cohort, might have been too low to act *in vivo* on P-gp expression.

It is clearly established that CKD greatly influences the nonrenal clearance of many drugs, justifying dosing adjustment.[4–6,11,63] Because of their importance in the overall elimination of drugs, transporters are decisive, even for drugs that are primarily metabolized by enzymes. However, drug distribution can also be affected by other factors, such as the drug's physical and chemical characteristics, plasma protein binding, organelle sequestration, individual genetic conditions, and nutritional status. All of these factors may influence the pharmacokinetics of drugs, and they are exactly what makes it difficult to identify specific molecular mechanisms in patients.

In conclusion, we provide evidence that P-gp may be involved in an AhR-dependent detoxification process induced in response to the uremic toxin IS. It could lead to a possible subsequent reduction in the intracellular accumulation of P-gp substrates and changes in their elimination rates. We propose that the altered clearance of drugs associated with CKD could be due to the effects of some uremic toxins on transporter expression and activity. It is important to identify

the transporters affected during CKD and the factors that can modulate their activities to improve the use of drugs in patients with CKD and reduce their secondary effects.

CONCISE METHODS

Cell Culture and Treatments

HepG2 cells obtained from hepatocellular carcinoma (ATCC, Life Technologies, Saint Aubin, France) were grown in α-MEM (Life Technologies) containing 10% premium FBS (Life Technologies) and 400 mg/L of G418 (Geneticin; Life Technologies) under standard cell culture conditions (humidified atmosphere, 5% CO_2, and 37°C). IS (Sigma-Aldrich, Saint Quentin Fallavier, France) was diluted from a stock solution prepared in water. The concentrations of IS were 0.01, 0.03, 0.08, 0.2, 0.4, and 1 mM, and they were in the range of those found in patients with CKD.[28] Because IS is provided in a potassium salt form, we used KCl at equivalent concentrations as controls other than culture medium alone. We found no differences between cells treated with culture medium alone or KCl. For some experiments, 40 g/L of human albumin (VIABELEX; Laboratoire LFB Biomédicaments, Les Ulis, France) was added to the culture medium to mimic the *in vivo* conditions of human albumin blood concentration. For the incubation of 96 hours, the medium was changed three times. Three samples of human primary hepatocytes were purchased from Lonza (Levallois, France) and used according to the manufacturer's instructions. Briefly, the cells were rapidly thawed and seeded at 10^6 cells per 1 ml for 2 days. The medium containing IS or KCl was then added for 16 hours. TCDD (Sigma-Aldrich), an AhR agonist, diluted in DMSO (Sigma-Aldrich) was used at 30 nM and compared with medium containing DMSO. Cells were treated during the indicated times.

Mice

C57BL/6J wild-type mice were purchased from The Jackson Laboratory and maintained as a breeding colony in the animal care facility at the Faculty of Medicine of Marseille. Male mice with CKD were used in this study. At 13 weeks of age, the mice were fed *ad libitum* with a 0.25% adenine-enriched diet (A04+0.25% adenine; SAFE, Augy, France) or a regular chow diet (A04 standard; SAFE) as controls. After adenine was administered for 2 weeks to induce uremia,[30] it was withdrawn, and mice were fed with the regular chow diet for another week. By hematoxylin and eosin staining of kidney and liver sections (5 μm), we observed the presence of 2,8-dihydroxyadenine crystals in the kidney but did not in the liver of mice fed with adenine (Supplemental Figure 3) as previously described.[32]

As a second model of CKD, we performed 5/6 nephrectomy using a two-step procedure with minor modifications as previously described.[31] Briefly, mice (9 weeks old) underwent 5/6 nephrectomy or sham surgery under general anesthesia (ketamine 125 mg/kg, xylazine 12.5 mg/kg, and atropine 0.25 mg/kg intraperitoneally). The left kidney was exposed through a flank incision, and we applied cortical electrocautery to the upper and lower poles (two thirds of the left kidney). One week later, we performed right total nephrectomy through a similar incision. Mice were euthanized at 6 weeks after induction of CKD. Sera were obtained from blood withdrawn *via* cardiac puncture during euthanasia. Creatinine and urea levels were measured with an Olympus AU400 auto analyzer at the Biochemistry Laboratory of the Centre de Recherche sur l'Inflammation (UMR 1149 INSERM, Université Paris Diderot, ERL CNRS 8252, Paris, France).

Total RNA Extraction from HepG2 Cells and Mouse Livers

Total RNA was extracted from HepG2 by RNeasy Mini Kit (Qiagen, Courtaboeuf, France). Mouse livers were lysed in TRIzol, and total RNA was purified by chloroform extraction and isopropanol precipitation. RNA concentration was determined using a NanoDrop Spectrophotometer (Thermoscientific, Wilmington, DE).

Comparative Quantification of mRNA Levels

Gene expression was analyzed by reverse transcription and comparative PCR. To analyze mRNA levels in HepG2 cells, primers were designed by the software primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to systematically encompass an intron to avoid amplification of contaminating genomic DNA. Reverse transcription using random primers and oligodT was performed on 500 ng of total RNA of each sample using the Takara PrimeScript RT Reagent Kit (Ozyme, Saint Quentin en Yvelines, France) followed by PCR on 12.5 ng of cDNA using the Takara SYBR qPCR Premix Ex Taq (Ozyme). PCR reactions were made with Mx3000P (Agilent, Massy, France) using comparative quantification of mRNA levels. All PCR reaction efficiencies were determined with Mx3000P software, and they were always superior to 90%. The results shown in this study were normalized with *HPRT* (housekeeping gene). The fusion curves were analyzed to assess the specificity of detected fluorescence. The sequences of the primers for *HPRT1* and *AhR* are listed below:

HPRT (*HPRT1*; HGNC:5157)
HPRT-F 5′GGATTATACTGCCTGACCAAGGAAAGC3′
HPRT-R 5′GAGCTATTGTAATGACCAGTCAACAGG3′
AhR (*AhR*; HGNC:348)
AhR-F 5′TGTTGGACGTCAGCAAGTTC3′
AhR-R 5′TGGTGCCCAGAATAATGTGA3′.

The primers specific for transporters (SLC10A1, SLC22A1, SLC22A7, SLC47A1, SLCO1B1, SLCO1B3, SLCO2B1, ABCB1, ABCB11, ABCC2, ABCC3, ABCC4, ABCC6, and ABCG2) are listed in Supplemental Table 1.

To analyze mRNA levels in mouse livers, reverse transcription was performed from 500 ng of total RNA with the Takara PrimeScript RT Reagent Kit. Primers and MGB-Taqman probes were purchased from Life Technologies (*abcb1a*: Mm00440761_m1; *abcb1b*: Mm00440736_m1; and the housekeeping gene *Gusb*: Mm01197698_m1). The PCR reaction mixture was prepared using the Brilliant II QPCR Master Mix (Agilent Technologies, Les Ulis, France). All PCR reactions were performed with the Mx3000P. The data were acquired and analyzed with the MxPro software (Agilent Technologies). Target gene expression was normalized on the basis of the *Gusb* content of each sample, and it was subsequently normalized to a basal mRNA level with the equation: N target $= 2\Delta Ct$ sample, where ΔCt is the Ct value of the target gene minus the Ct value of

the *Gusb* gene. The results are reported as normalized mRNA levels (*i.e.*, the *N* target value divided by the *N* target value of the smallest quantifiable amount of target gene mRNA [target gene Ct value =35]).

Uremic Toxins Measurement

IS was measured in human serum by HPLC using a reversed phase column, an ion-pairing mobile phase, and an isocratic flow as described.[28] The sera of patients were collected, processed, and stored at −80°C until determination of uremic toxin concentrations.

R123 Accumulation Assay

Cells were cultured in the 24-well plates with a density of 15×10^4 per well for 24 hours. Then, cells were treated overnight with KCl or IS. Cells were washed once with HBSS; then, they were incubated for 5 minutes with HBSS containing or not containing 5 μM R123, a fluorescent substrate for P-gp.[64] Cells were washed twice with HBSS in the presence or absence of the P-gp inhibitors verapamil and elacridar at 50 and 2 μM, respectively. Then, cells were subsequently incubated with HBSS with or without the P-gp inhibitors for 30 minutes. Cells were washed once with cold HBSS before the addition of 200 μl enzyme-free dissociation buffer at 37°C for about 2 minutes. The dissociated cell suspensions from each well were then placed in 15-ml tubes and subjected to centrifugation at 4°C for 3 minutes. The supernatant was discarded, and the cell pellet was reconstituted in ice cold HBSS-0.1% FBS for flow cytometric analysis immediately using a Gallios Flow Cytometer (Beckman-Coulter, Roissy, France).

Protein Extraction from HepG2

Cells were cultured in the T75 flasks with a density of 1×10^6 per flask for 24 hours. Then, cells were treated overnight with IS or TCDD and their controls KCl or DMSO, respectively. Cell culture flasks were placed in ice and washed once with cold PBS. Then, 500 μl cold RIPA buffer (50 mM Tris·HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 1 mM EDTA, pH 7.4) containing protease inhibitors (Complete Mini; Roche Diagnostics France, Meylan, France) was added and kept at 4°C for 30 minutes. Cells were scraped, transferred to a microfuge tube, and maintained at constant agitation for 30 minutes at 4°C. Samples were sonicated for 1 minute and then kept on ice for another 1 minute between each pulse; this procedure was repeated three times. The homogenate was then centrifuged at $12,000 \times g$ for 15 minutes at 4°C, and the supernatant was collected. Protein concentrations were measured with the Bicinchoninic Acid Kit for Protein Determination (BCA1; Sigma-Aldrich).

Western Blot Analyses

Equal amounts of total protein (40 μg) from cell lysates were loaded on 4%–12% SDS-polyacrylamide electrophoretic gel and transferred onto a polyvinylidenedifluoride membrane. Nonspecific binding was blocked by immersing the membrane in PBS-5% BSA at room temperature for 1 hour. After saturation, the membrane was incubated overnight at 4°C with a primary antibody directed against P-gp (1:500 dilution; C219; Life Technologies) and actin (1:1000 dilution; D6A8; Cell Signaling, Yvelines, France) that was used as a loading control. After washing with PBST (PBS and 0.1% Tween-20) and PBS-5% BSA, the membrane was further incubated with the secondary

peroxidase-conjugated antibody (Beckman-Coulter) at a 1:2000 dilution for 1 hour at room temperature. Membranes were washed with 5% BSA, and revelation was made by chemiluminescence (ECL Western blotting substrate; Pierce, Courtaboeuf, France); the gel image was captured using the Syngene GBox (Ozyme). Densitometry analyses of chemiluminescence staining were performed with the software geneSys (Ozyme). Results were expressed as a ratio between values obtained with IS or TCDD and the value obtained with control, and then, they were normalized with values obtained with actin.

Flow Cytometry Analyses of P-gp Expression

HepG2 were detached with a prewarmed cell dissociation buffer (Cell Dissociation Buffer, enzyme free, HBSS; Life Technologies) for 3 minutes at 37°C. Cells were washed with HBSS-20% FBS and saturated with HBSS-3% BSA-0.2% saponine for 15 minutes at 4°C. Cells were centrifuged and then incubated for 1 hour at 4°C with mAb against P-gp diluted at 20 μg/ml or irrelevant control mAb at 20 μg/ml in HBSS-1% BSA-0.2% saponine. After being washed three times with HBSS-1% BSA, cells were analyzed using a Gallios flow cytometer. Median fluorescence intensity on the whole cell population was determined by the Kaluza software (Beckman-Coulter) and expressed in arbitrary units. The median fluorescence intensity measured on cells labeled with the mAb against P-gp was corrected with isotype control value.

Gene Silencing

Downregulation of *AhR* mRNA levels was achieved by using a pool containing the three siRNAs directed against AhR (1200, 1999, and 1998; 40 pmol each; Applied Biosystems, Courtaboeuf, France) and the Cell Line Nucleofector Kit V (Lonza) according to the manufacturer's instructions. Culture medium was changed 7 hours after the transfection. Because the decrease in *AhR* mRNA level was achieved 24 hours after transfection and remained stable until 96 hours, the effects of IS on *AhR* mRNA, protein, and activity were investigated 48 hours after transfection.

Patients

Patients were recruited in the Clinical Center of Investigation (APHM, Hôpitaux de Marseille, Marseille, France). We studied 109 patients with CKD (eGFR below 60 ml/min per 1.73 m^2) and kidney or heart transplantation under immunosuppressive treatment with cyclosporin. Patients with kidney transplantation (n=62) are a subgroup of the FEG cohort, and patients with heart transplantation (n=47) are a subgroup of GRATEC cohort. The characteristics of patients are listed in Table 2.

Study Approval

All participants gave their written informed consent before any study procedure. The study was approved by the local ethics committees and conforms to the principles outlined in the Declaration of Helsinki. The animal experiments conform to Directive 2010/63/EU of the European Parliament and were approved by the local ethics committee (Comité d'Ethique en Expérimentation Animale de Marseille; C2EA-14).

Statistical Analyses

Data are expressed as mean±SEM or median and interquartile range (25th–75th percentiles) according to the normality of their distribution.

For *in vitro* experiments, significant differences were determined using the Wilcoxon signed rank test or *t* test (GraphPad Software Inc., San Diego, CA). In mice experiments, significant differences were revealed by Mann–Whitney *U* test. Linear regression analysis was used to assess the relationship between serum cyclosporin dosage and levels of IS (Stata Software, Lakeway Drive, TX). A *P* value below 0.05 was considered statistically significant.

DISCLOSURES

None.

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