Remote sensing and signaling in kidney proximal tubules stimulates gut microbiome-derived organic anion secretion

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Membrane transporters and receptors are responsible for balancing nutrient and metabolite levels to aid body homeostasis. Here, we report that proximal tubule cells in kidneys sense elevated endogenous, gut microbiome-derived, metabolite levels through EGF receptors and downstream signaling to induce their secretion by up-regulating the organic anion transporter-1 (OAT1). Remote metabolite sensing and signaling was observed in kidneys from healthy volunteers and rats in vivo, leading to induced OAT1 expression and increased removal of indoxyl sulfate, a prototypical microbiome-derived metabolite and uremic toxin. Using 2D and 3D human proximal tubule cell models, we show that indoxyl sulfate induces OAT1 via AhR and EGFR signaling, controlled by miR-223. Concomitantly produced reactive oxygen species (ROS) control OAT1 activity and are balanced by the glutathione pathway, as confirmed by cellular metabolomic profiling. Collectively, we demonstrate remote metabolite sensing and signaling as an effective OAT1 regulation mechanism to maintain plasma metabolite levels by controlling their secretion.

kidney proximal tubule | organic anion transporter 1 | remote sensing and signaling | indoxyl sulfate

The human gut provides habitat for a complex indigenous microbial ecosystem, governed by an astonishing number of microbial genes (1). This genetic capital, often referred to as the microbiome, codes for an intricate web of metabolic capacities that supplements our mammalian metabolism (2). The ensuing metabolic symbiosis allows exploitation of nutrient sources that are otherwise inaccessible by human metabolism (3). There is significant cross-talk between the gut microbial metabolism and the human metabolism. Metabolites unique to microbial metabolism enrich the human metabolome, thereby providing energy, vitamins, and trophic signals (2). From an evolutionary viewpoint, the human gut microbial ecosystem symbiosis provides mutualistic metabolic benefits, thus contributing toward organismal fitness (4).

This does not necessarily imply that each and every microbial metabolite is beneficial. On the contrary, most microbial metabolites undergo intense phase II metabolism, and numerous metabolites are actively excreted from the body (5, 6). The kidneys perhaps are the most important excretory route, and significant amounts of microbial metabolites can be found in the urine. This has fueled the hypothesis that the kidney excretory capacity is an essential part of the human microbial symbiosis. It allows for intestinal absorption of a wide array of mostly beneficial microbial metabolites, while the kidneys remove the fraction of metabolites that is useless and potentially deleterious. This interorgan communication via small molecules has been postulated as remote sensing and signaling (7). Ample evidence in patients with chronic kidney disease (CKD) provides strong support for the role of the kidneys in human microbial symbiosis (8, 9). A number of microbiota-derived metabolites, including indoxyl sulfate (IS), p-cresyl sulfate, and trimethylamine oxide, were found to accumulate in the blood parallel to the loss of kidney function, and proven to be associated with clinical outcomes in patients with CKD (10, 11). This paradigm has been coined the gut–kidney axis.

Remote metabolite sensing and signaling is a mechanism to minimize perturbations of body homeostasis due to environmental metabolic challenges (7, 12, 13). Coordinated adjustment

Significance

Here, we report that the kidney has an effective sensing and signaling mechanism to balance microbial metabolite levels in the human body. Using healthy volunteers and in vivo and in vitro assays, we show that the kidney responds to elevated endogenous metabolite levels and stimulates organic anion secretion by AhR and EGFR signaling, controlled by miR-223 and reactive oxygen species. This remote sensing function aids body homeostasis and has important implications for the identification of novel therapeutic targets to preserve kidney function in patients.

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of transporter networks with overlapping substrate preferences have been described for glucose, amino acids, and lipids in the liver, the intestines, and the brain (14–16). Membrane transporters have been postulated to be involved in metabolite sensing and are widely expressed in epithelial barriers, including the kidney proximal tubule segment (17). The ability of the organic anion transporters (OAT), member of the renal solute carrier 22 family, to transport a wide spectrum of waste solutes including those derived from digested food and endogenous metabolic processes, but also environmental compounds and drugs, make them plausible candidates for remote sensing (12, 14, 18–20).

We investigated whether kidney proximal tubules can regulate body homeostasis by sensing elevated endogenous gut microbiome-derived metabolites levels and can respond by stimulating secretory mechanisms through OAT1 (*SLC22A6*), for which we studied IS as prototypical toxin. We demonstrate the existence of a metabolite sensing and signaling mechanism in the kidney governed by epidermal growth factor receptor (EGFR) and OAT1 as IS sensors, and the interplay of the aryl-hydrocarbon receptor (AhR)–ARNT complex and downstream EGFR MAPK–ERK pathways, accompanied by miR-223 and reactive oxygen species (ROS) signaling. We here describe the ability of the kidney tubule to sense elevated metabolite levels and respond by activating a pathway that boosts metabolite secretion. The ability for remote sensing and signaling of gut microbiota-derived metabolites by the kidney further supports the gut–kidney axis in symbiosis.

Results

Kidneys Sense and Signal Elevated Gut-Derived Metabolite Levels and Induce Renal Secretion. We previously studied the impact of high-protein diet on tryptophan and phenolic metabolites (21). Human volunteers subjected to a high-protein diet for 14 d (Fig. 1*A*) had elevated plasma levels of IS (21), accompanied by an average increased urinary IS excretion of 45 ± 14% (Fig. 1*B*, $P < 0.01$). Concentration-dependent augmentation of transporter-mediated urinary excretion is not uncommon as long as serum concentrations are below transport maximum. However, we questioned whether the excretory route for IS can be sensed and regulated to balance serum concentrations. In this exploratory study, we observed a 70% increase of OAT1 messenger RNA (mRNA) expression in renal proximal tubule epithelial cells isolated from urine, although this increase was not significant (Fig. 1*C*). As recently shown by our group and others, urinary epithelial cells are viable cells showing proximal tubule characteristics similar to renal epithelial cells isolated from human kidney tissue (22, 23), emphasizing that urine is an ideal source for studying OAT1 expression in our cohort. To further explore whether the excretory route for IS can be sensed and regulated, we analyzed data from a second human intervention study ($n = 36$) subjected to protein concentrates extracted from corn, whey, and bovine plasma (Fig. 1*D*). Tryptophan and tyrosine, precursors of IS and p-cresyl sulfate, are expected to be most abundantly present in animal-derived diets (24). Indeed, urinary IS and p-cresyl sulfate excretion were increased following bovine-derived protein intake compared with corn- and whey-derived proteins (Fig. 1 *E* and *F*), although p-cresyl sulfate increased to a lesser extent compared with IS.

To test whether IS itself regulates OAT1 expression, we used an adenine CKD rat model gavaged with IS (Fig. 1*G* and *SI Appendix*, Table S1). Compared with the vehicle group, renal IS clearance was increased 7 wk after IS treatment (4.4 ± 0.7 mL min^{-1} vs. control 0.1 ± 0.02 mL min^{-1}; Fig. 1*H*), and OAT1 mRNA expression isolated from urinary kidney epithelial cells was 2.7 ± 0.6-fold induced (Fig. 1*I*, $P < 0.05$). Similarly, CKD rats were gavaged with p-cresyl sulfate to test metabolite sensing and signaling specificity. Clearance of both metabolites, p-cresyl sulfate and IS, was deteriorated after 5 wk of p-cresyl sulfate administration compared with week 1 treatment (*SI Appendix*, Fig. S1 *A* and *B*). The OAT1 protein expression was over 40% reduced in the p-cresyl sulfate group, whereas expression levels were maintained in the IS group (*SI Appendix*, Fig. S1*C*). Together, the IS-mediated induction of the OAT1 secretion pathway suggests the involvement of an active and specific metabolite sensing and signaling mechanism.

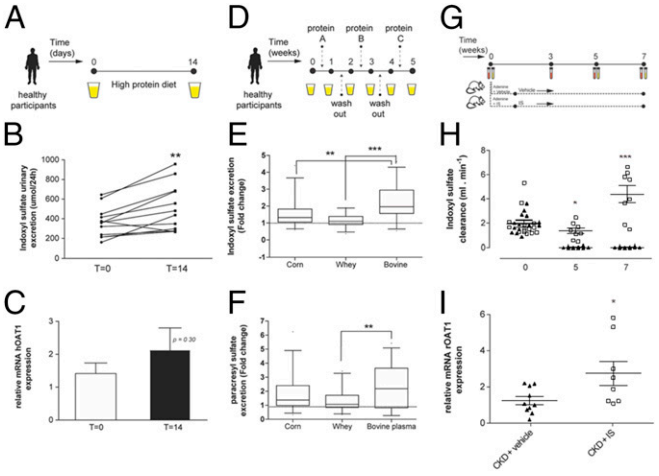

Fig. 1. Human (h) and rat (r) kidneys sense and signal elevated gut-derived metabolite plasma levels and induce their secretion. (*A*) Schematic diagram of the human study design. (*B*) IS excretion before and after high-protein diet intervention. (*C*) Relative mRNA hOAT1 expression before and after high-protein diet intervention in human kidney cells. (*D*) Schematic diagram of the second human study ($n = 36$ volunteers) using protein concentrates extracted from corn, whey, and bovine plasma in a randomized manner. (*E*) IS and (*F*) p-cresyl sulfate excretion following corn, whey, and bovine plasma protein concentrate intervention. (*G*) Schematic diagram of the animal study design. (*H*) IS clearance over time in vehicle (triangle) and IS (square)-treated CKD rats. (*I*) Relative mRNA rOAT1 expression in vehicle (triangle) and IS (IS, square)-treated rat kidney cells. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

IS Induces OAT1 Expression and Function via the AhR and EGFR Axis. To determine the mechanism of IS-mediated OAT1 induction, we explored the involvement of the AhR–ARNT complex, the EGFR pathway, and associated molecules. It has been shown that IS induces the AhR, a ligand-dependent transcription factor (25, 26). In turn, AhR induces the expression of membrane transporters belonging to the ATP binding cassette family. Indeed, we observed that dietary protein-derived metabolites activated AhR, and that IS appeared to be the most potent ligand (*SI Appendix*, Fig. S2 and Table S2). Exposure of human primary kidney epithelial cells or mature immortalized human proximal tubule epithelial cells (ciPTEC) (27) to a clinically relevant IS concentration (Fig. 2*A*) resulted in a robust increase of OAT1 expression that could be inhibited by AhR antagonists (Fig. 2 *B*–*D*). In addition to activating the AhR transcription factor, IS binds to the extracellular domain of the EGFR (28), a receptor previously implicated in OAT1 regulation as well (29). Indeed, inhibition of EGFR by cetuximab, a blocking monoclonal antibody, attenuated the IS-mediated induction of OAT1, thereby confirming its role in sensing and signaling (Fig. 2*D*). In addition, IS-induced OAT1 transport activity was inhibited by AhR- or EGFR-selective antagonists (Fig. 2*E*), sensitive to blocking transporter-mediated intracellular accumulation of IS (*SI Appendix*, Fig. S3), and the presence of EGF is essential to stimulate IS-induced OAT1 transport activity (Fig. 2*F*). Together, our data demonstrate that IS activates OAT1 expression and transport via the EGFR sensor and AhR signaling.

EGFR stimulation leads to activation of downstream MAPK and NF-κB signaling pathways, as shown on mRNA (Fig. 2*G* and

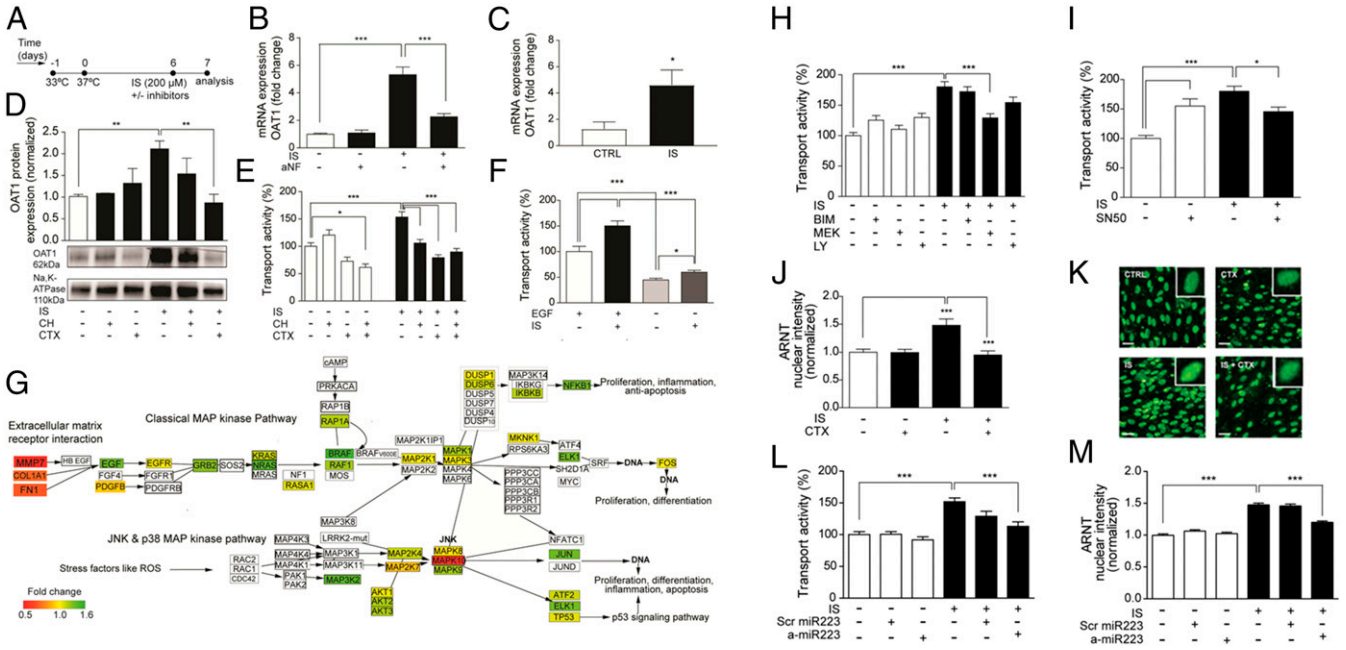

Fig. 2. IS induces OAT1 expression and function in vitro via the AhR and EGFR axis under the control of miR-223. (*A*) Schematic diagram of the experimental in vitro design using renal proximal tubule cells. (*B*) Relative mRNA hOAT1 gene expression in control and IS treated ciPTEC cells in the presence or absence of alpha-naphtoflavone (aNF) and (*C*) in primary kidney cells. (*D*) Relative OAT1 protein expression corrected for loading control using Na,K-ATPase and normalized to control. Control and treated cells in the presence or absence of CH-223191 (CH) and cetuximab (CTX) are shown. (*E*) OAT1 activity monitored using fluorescein transport in control and treated cells in the presence or absence of CH and CTX and normalized to control. (*F*) Fluorescein transport in control and treated cells in the presence or absence of EGF. (*G*) Relative mRNA expression EGF signaling in IS-treated cells compared with control. White boxes are not measured. For complete result list of genes tested, see *SI Appendix*, Table S3. (*H*) Fluorescein transport in control and treated cells in the presence or absence of bisindolylmaleimide (BIM; protein kinase C inhibitor), MEK (U-0126; MEK inhibitor), and LY-294002 (LY; phosphoinositide 3-kinase inhibitor) and normalized to control. (*I*) Fluorescein transport in control and treated cells in the presence or absence of SN50 trifluoroacetate salt (SN50; NF-κB inhibitor) and normalized to control. (*J* and *K*) Nuclear ARNT expression in control and treated cells in the presence or absence of CTX. Representative images are shown in *K*. (*L*) Fluorescein transport in control and treated cells in the presence or absence of scrambled antagomiR-223 (Scr miR223) or antagomiR-223 (a-miR223) and normalized to control. (*M*) Nuclear ARNT expression in control and treated cells in the presence or absence of Scr miR223 or a-miR223 and normalized to control. Representative images are shown in *SI Appendix*, Fig. S4. Data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. (Scale bar: *K*, 10 μm.)

SI Appendix, Table S3) and functional transport level using MAPK and NF-κB inhibitors (Fig. 2 *H* and *I*). When EGFR signaling is inhibited by cetuximab, the nuclear expression of ARNT, the counter complex of AhR to which intracellular IS binds after uptake, is diminished (Fig. 2 *J* and *K*). This confirms the cross-talk between EGFR and AhR in IS sensing and signaling. Furthermore, microRNA-223 (miR-223) was reported to regulate genes associated with both AhR and EGFR signaling and might play a pivotal role in the kidney sensing and signaling hypothesis (30, 31). The cross-talk is indeed coregulated by miR-223, as its pharmacological inhibition reduced OAT1 activity (Fig. 2*L*) and deteriorated ARNT nuclear expression (Fig. 2*M* and *SI Appendix*, Fig. S4). Concomitantly, miR-223 expression itself was not altered in treated cells, and scavenging miR-223 did not influence MAPK1, BRAF (EGF signaling), or AhR and AhR repressor gene expressions (*SI Appendix*, Fig. S5). These data show that the interrelation between multiple signaling pathways is key in OAT1 regulation during the metabolite sensing and signaling adaptation.

A 3D physiological kidney model that we recently established allows polarization of the renal epithelial cell layer by seeding the cells onto a hollow-fiber scaffold (Fig. 3*A* and refs. 32 and 33). Using this model, we were able to demonstrate that transepithelial IS secretion is enhanced after IS treatment (35% ± 13; Fig. 3*B*). This is the net effect of induced OAT1 function (Fig. 2*E*), which was accompanied by an enhanced expression of the efflux transporters breast cancer resistance protein and multidrug resistance protein 4 (*SI Appendix*, Fig. S6); both are involved in luminal secretion of IS (34) and regulated by EGFR signaling (29).

Reactive Oxygen Species Are Driving Forces in Remote Sensing and Signaling and Are Efficiently Detoxified by Glutathione Metabolism. AhR activation and its nuclear translocation have been associated with cellular stress and the production of ROS (35, 36). We confirmed that ROS levels were induced by IS (Fig. 4*A*). Further, we show that ROS serve as important messengers to stimulate OAT1 function, as ROS quenching by antioxidants, using trolox or acetyl cysteine, counteracted the IS-induced transport effect (Fig. 4*B*). Metabolomic profiling to further explore intracellular metabolic changes upon IS stimulation on renal epithelial cells revealed changes in glutathione and beta-alanine metabolism (Fig. 5 and *SI Appendix*, Table S4). Glutathione is an important antioxidant, critical for protecting cells from oxidative stress. The ratio of reduced glutathione to oxidized glutathione is a good

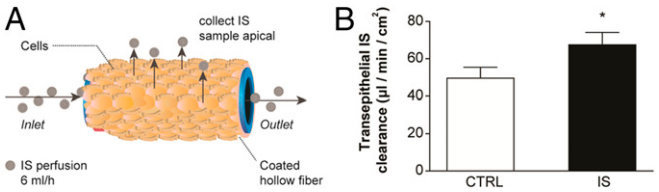

Fig. 3. Ameliorated transepithelial transport using IS exposure in a 3D kidney model. (*A*) Schematic showing the experimental design of transepithelial transport across a 3D bioengineered kidney tubule. (*B*) IS clearance in control and IS-treated bioengineered kidney tubules. Data are presented as mean ± SEM. **P* < 0.05.

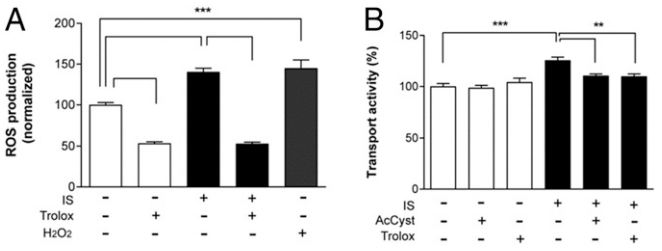

Fig. 4. Reactive oxygen species aid remote metabolite sensing and signaling. (*A*) ROS production in control and treated cells in the presence or absence of trolox. Data were normalized to control. (*B*) Fluorescein transport in control and treated cells in the presence or absence of *N*-acetyl-l-cysteine (AcCyst) and trolox. Data were normalized to control and are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$.

measure of oxidative stress and was previously shown to be reduced in IS-treated endothelial cells (37). Consistent with these findings, IS-treated renal epithelial cells demonstrated a reduced relative ratio as well (ratio of 0.8 ± 0.05, $P = 0.03$), further confirming that IS induces oxidative stress in these cells (Fig. 5*A*). In addition, beta-alanine metabolism is an indicator of increased cellular oxygen consumption, and its declined levels in IS-treated cells are another indicator of oxidative stress (Fig. 5). Altogether, cellular stress responses during remote sensing and signaling are tightly regulated, and ROS molecules control OAT1-mediated transport.

Discussion

It is hypothesized that kidney function is an essential part of human gut microbiome symbiosis. The kidney's excretory capacity of unusable (potentially deleterious) microbial metabolites is unmatched by other organs. Here, we describe the identification of an effective mechanism by which human kidneys sense elevated IS levels through receptor-mediated signaling, and respond by inducing their secretory pathway via OAT1. This biological response in remote metabolite sensing and signaling is governed by the complex interplay between OAT1, EGFR, AhR, and miR-223 that induces ARNT translocation and ROS-associated signal transduction. Together, the regulation pathway reveals a detoxification mechanism facilitated by kidney epithelial cells to remove gut-derived metabolites and to aid body homeostasis.

As presented here, EGFR activation by IS and downstream MAPK–ERK signaling plays a pivotal role in ARNT nuclear translocation in kidney epithelial cells. Our results are supported by previous findings by Tan et al. (38), who showed that MAPK–ERK signaling is activated by dioxin, a known AhR ligand, and that this activation potentiates the transcriptional activity of AhR–ARNT heterodimers in mouse hepatoma cells. In addition, EGF supplementation stimulated the binding of the ARNT complex to a responsive element within the cyclooxygenase-2 gene promoter region in human squamous cell carcinoma cells (39), molecular docking studies revealed that IS binds to the extracellular domain of EGFR (28), and EGFR-dependent regulation of

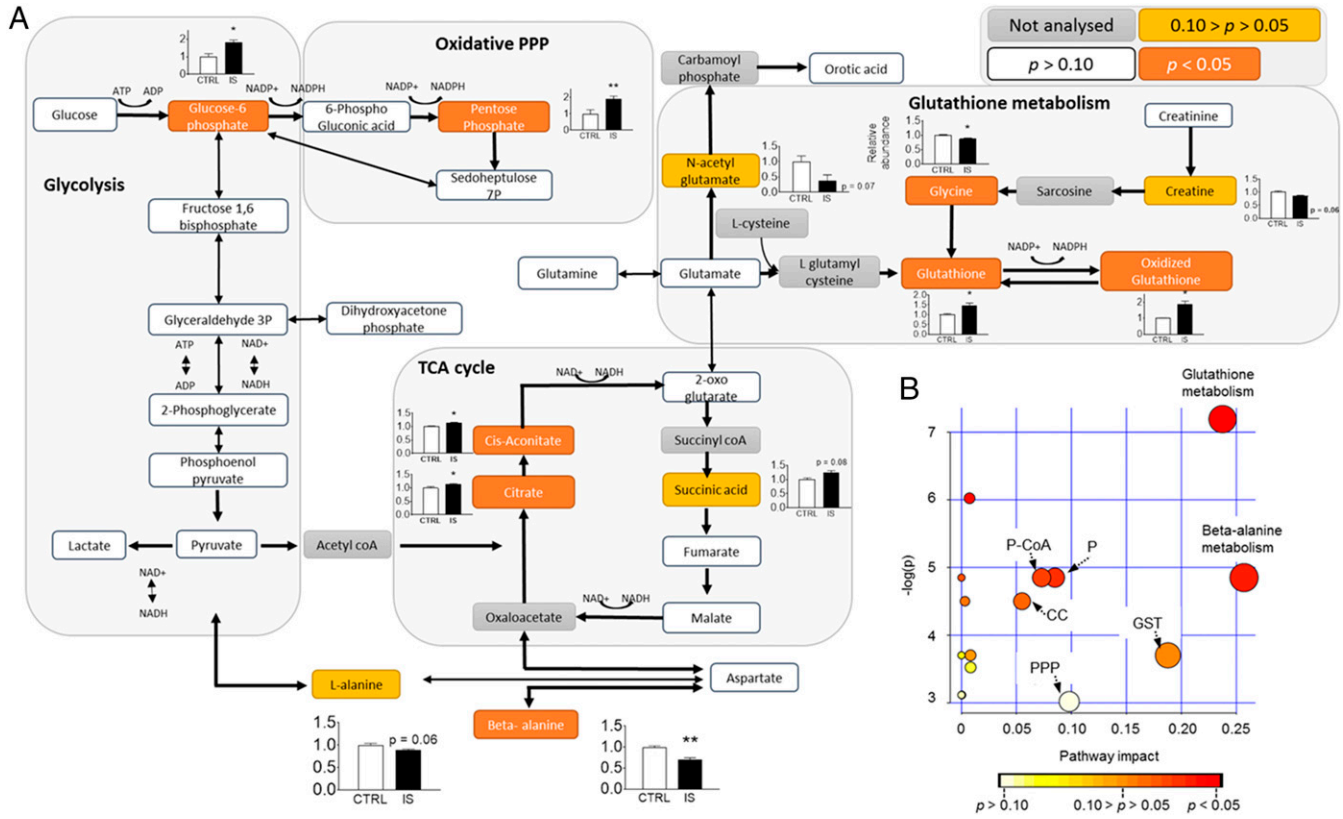

Fig. 5. Induced glutathione and reduced beta-alanine metabolism during IS sensing and signaling in response to oxidative stress. (*A*) Metabolomic analysis of IS-treated cells compared with control. Relative metabolite abundance of the glutathione metabolism, TCA cycle, urea cycle, oxidate pentose phosphate pathway (PPP), and glycosis are plotted. Color code: orange, $P < 0.05$; yellow, $0.10 > P > 0.05$; white, $P > 0.10$; gray, not analyzed. (*B*) Pathway analysis based on impact and *P* value showing that glutathione metabolism is enhanced and beta-alanine is reduced in IS-treated cells compared with control. Larger circles farther from the *y* axis and orange-red color show higher impact of pathway. P-CoA, pantothenate and co-A biosynthesis; P, propanoate metabolism; CC, citrate cycle; GST, glycine, serine, and threonine metabolism. $^{*}P < 0.05$ and $^{**}P < 0.01$.

OAT1 via PI3K-AKT and MAPKK–ERK signaling was demonstrated in cetuximab-treated renal epithelial cells (29).

We provide direct evidence that IS activates EGFR and downstream MAPK signaling, which stimulates AhR–ARNT nuclear translocation and results in enhanced OAT1 expression and function in the kidney. Concomitantly, we investigated related signaling factors including miR-223 and ROS. To date, over 200 targets have been described for miR-223 (40), emphasizing its ubiquitous involvement in cellular processes. Scavenging miR-223 leads to a decrease in ARNT translocation, stressing that miR-223 is a positive posttranscriptional regulator of ARNT protein expression. The link between miR-223 and ARNT was also detected in macrophages by Ogando et al. (31), but they identified miR-223 as a negative ARNT regulator. In our study, miR-223 might control a repressor related to the AhR–ARNT complex; however, exact target identification would require further research.

The balance between oxidation and antioxidation is essential for many biological processes. It is well documented that IS induces cell stress, ROS production, and inflammation in many cell types, including endothelial cells, muscle cells, cardiomyocytes, and renal epithelial cells (37, 41–43). On the other hand, ROS can act as secondary messengers in key physiological pathways including AhR, EGFR, and NF-κB signaling (36, 44, 45), suggesting their auxiliary role in signal transduction during metabolite sensing. Our metabolomics analysis showed a clear cellular stress response upon IS treatment, similar to previous studies in, for example, skeletal muscle cells (41). Interestingly, the generation of ROS is one of the driving forces in the metabolite sensing and signaling events, as ROS scavenging counteracted the IS-induced up-regulation of functional OAT1. Thus, antioxidative response pathways and ROS levels in renal epithelial cells emphasize the importance, and also the complexity, of small-molecule communication within the kidney epithelium.

Our work fits in the "remote sensing and signaling hypothesis" as described by Nigam and coworkers (7, 17, 20, 46) for the solute carrier family of transporters in mediating interorgan and even interorganismal communication. These transport proteins are marked by their broad substrate specificity and ubiquitous expression in tissues and organs with a barrier function. The network regulates the transport of important metabolites and signaling molecules across organs (e.g., gut–liver–kidney axis) by tuning the expression and function of these drug transporters accordingly (46). Here, we demonstrate that the microbial metabolite indole, which is oxidized and conjugated with sulfate in the liver to IS, acts as a signaling molecule to stimulate the expression and function of uptake and efflux transporters involved in its renal excretion. In the kidney, AhR-mediated detoxification mechanisms fulfill a central role in stimulating the clearance of endobiotics and xenobiotics, but also of environmental pollutants (47). For example, dioxin-liganded AhR binding massively upregulates cytochrome P450s (CYP) enzymes. Consequently, dioxin is favorably metabolized by CYPs and efficiently eliminated from the body, emphasizing that multiple mechanisms can be activated to stimulate waste clearance. Altogether, remote sensing and signaling orchestrates the efficient removal of gut-derived metabolites to aid body homeostasis.

The sensing mechanism elicits the possibility to modulate OAT1 function in the kidney and may lead to a new era of potential therapeutic avenues to preserve kidney function upon injury. OATs are also expressed in other organs (e.g., liver, brain), but the abundant and predominant expression of OAT1 in the kidney gives rise to opportunities for kidney-specific targeting. In support, OAT1 expression is maintained in CKD rats administered with IS, whereas CKD rats gavaged with p-cresyl sulfate showed reduced OAT1 expression and clearance of IS and p-cresyl sulfate. In addition, the development of cell-based regenerative therapies to replace kidney function, like the bioartificial kidney (i.e., waste removal facilitated by kidney epithelial cells), is currently of high interest worldwide (33, 48). Activating OAT1-mediated transport using the metabolite sensing mechanism in a cell-aided device would boost the transport capacity of these cells. Consequently, an advanced transport capacity will lead to enhanced waste solute removal critical to the successful development of cell-based therapies for kidney disease. Further, OAT3 contributes to the renal excretion of IS, although with a somewhat lower affinity, as shown for rat (49) and human (50) isoforms. Both solute transporters share common substrates and are regulated by similar pathways, but distinct regulatory mechanisms have been described as well (46, 51). Moreover, OAT3 has been proposed to mediate vascular toxicity through IS uptake and subsequent AhR/NF-κB signaling (52). A possible contribution of OAT3 to the microbiome metabolite sensing and signaling renal excretion pathway was beyond our interest, as we used sole OAT1-expressing renal cells (27), and has yet to be investigated.

In conclusion, we identified molecular targets involved in remote metabolite sensing and signaling that result in enhanced OAT1 function and consequently induce waste removal by the kidney. The cross-talk between AhR, EGF, and miR-223, accompanied by an altered redox status, is essential for this mechanism and emphasizes the well-defined regulation in kidney cells to modulate transport processes. These data provide additional support for the hypothesis that kidney function is an essential part of the human gut microbiome symbiosis, named the gut–kidney axis.

Materials and Methods

For details, see *SI Appendix, Supplementary Methods* (53–58).

Clinical Study I. The study in human volunteers was performed according to the Declaration of Helsinki and approved by the ethics committee of the University Hospitals Leuven, and written informed consent was obtained from all subjects.

Clinical Study II. The trial was carried out in accordance with the declaration of Helsinki and was approved by the medical ethical committee of Wageningen University. Signed informed consent was obtained from all subjects before initiation of the study. This trial was registered at ClinicalTrials.gov as NCT03744221.

Animal Experiments. Animal experiments were approved by the University of Antwerp Ethics Committee (Permit no. 2012-13) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals 85-23 (1996).

Cell Models and Ethics Statement. Kidney tissue was obtained from a nontransplanted donor, after giving informed consent, to isolate primary epithelial cells as outlined by Jansen et al. (18) and was approved by the medical ethical committee of Radboud University Medical Center. No clinical history of renal disorders or any other chronic disease was identified.

In Vitro Assays. For all in vitro experiments, cells were exposed to 200 μM IS, with or without inhibitors, in serum-free medium at day 6, 37 °C, 5% (vol/vol) CO_2 for 24 h. For details, see *SI Appendix, Supplementary Methods*.

Statistical Analysis. All data are expressed as mean ± SEM of 3 independent experiments performed at least in triplicate, unless stated otherwise. Statistical analysis was performed using 1-way or 2-way ANOVA analysis followed by Tukey or Sidak's posttest or, when appropriate, an unpaired *t* test with GraphPad Prism version 7. A P value of <0.05 was considered significant and indicated using one asterisk; ** = $P < 0.01$, and *** = $P < 0.001$.

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