Does indoxyl sulfate, a uraemic toxin, have direct effects on cardiac fibroblasts and myocytes?

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Aims
Indoxyl sulfate (IS) is a uraemic toxin found at high concentration in patients with chronic kidney disease (CKD) co-morbid with chronic heart failure (CHF). The aim of this study was to determine direct effects of IS on cardiac cells as well as the pro-inflammatory effect of IS.

Methods and results
Indoxyl sulfate significantly increased neonatal rat cardiac fibroblast collagen synthesis (by 145.7% vs. control, \( P < 0.05 \)) and myocyte hypertrophy (by 134.5% vs. control, \( P < 0.001 \)) as determined by \(^3\)H-proline or \(^3\)H-leucine incorporation, respectively. Indoxyl sulfate stimulated tumour necrosis factor-alpha, interleukin-6 (IL-6), and IL-1β mRNA expression in THP-1 cells as quantified by RT–PCR. Both p38 (RWJ-67657) and MEK1/2 (U0126) inhibitors suppressed all these effects by IS. Furthermore, western blot analysis showed that IS activated mitogen-activated protein kinase (MAPK) (p38, p42/44) and nuclear factor-kappa B (NFκB) pathways. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that IS exerted its effects without affecting cell viability.

Conclusion
This study has, for the first time, demonstrated that IS has pro-fibrotic, pro-hypertrophic, and pro-inflammatory effects, indicating that IS might play an important role in adverse cardiac remodelling mediated via activation of the p38 MAPK, p42/44 MAPK, and NFκB pathways. Targeting reduction of IS and/or the pathways it activates may represent a novel therapeutic approach to the management of CHF with concomitant CKD.

Keywords
Uraemic toxins • Fibrosis • Hypertrophy • Cytokines • Cell signalling

Introduction
A close relationship between chronic kidney disease (CKD) and chronic heart failure (CHF) has been demonstrated both clinically and using basic research methodologies. The relationship of these two entities is bi-directional with regard to physical, chemical, and biological mechanisms. Via a vast array of inter-related derangements, failure of one organ can worsen function of the other, which in turn further accelerates the progression of failure of both. Chronic kidney disease is an important contributor to cardiovascular mortality, which in turn is responsible for 40–50% of all deaths.¹ Acceleration of coronary artery disease and left ventricular hypertrophy (LVH) are major cardiac problems observed in CKD patients and may be contributory to the CHF that is frequently an accompaniment of these patients.²

There may also be additional factors contributing to CHF in CKD patients. In the setting of CKD, there is systemic accumulation of uraemic toxins many of which can be eliminated by dialysis. Removal of some toxins is, however, limited owing to their high protein-binding capacity.³ Many studies have investigated detrimental effects of uraemic toxins on various organs, the kidney itself in particular. However, effects on the heart have rarely been evaluated thus far.

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Indoxyl sulfate (IS) is one of a number of protein-bound uraemic toxins that accumulate in patients with CKD. Current conventional haemodialysis is ineffective at removing this toxin, as 90% of IS is bound to albumin and the IS–albumin complex molecule is larger than the dialysis membrane’s pore size. Thus, the balance of IS in free or protein-bound forms is about 1 to 9 in ratio, which provides a pool/reservoir of IS circulating even after dialysis in uremic patients. In CKD patients, the total IS concentrations vary and can be as high as 500 μM, whereas in healthy subjects concentrations are extremely low (<0.1–2.39 μM). The free form of IS is about 10% of the total IS in CKD patients; however, it is non-detectable in normal subjects.

Indoxyl sulfate has been shown to accelerate the progression of CKD owing to its pro-fibrotic effects in renal glomeruli and interstitium. Retained IS in renal failure is also associated with several detrimental effects on other organs such as altered thyroid function, endothelial dysfunction, vascular smooth muscle cell proliferation, and an increase in atherosclerosis in man. Cardiac effects of IS, thus far, have not yet been reported.

Fibrosis is a common finding in both CHF and CKD and is associated with the progression of both these diseases. Moreover, cardiac fibrosis as well as cardiomyocyte hypertrophy has been demonstrated in renal failure patients and experimental models of CKD. We therefore hypothesized that IS, increased in CKD, may have pro-fibrotic and pro-hypertrophic effects on cardiac cells, which may adversely contribute to cardiac remodelling.

Pro-inflammatory cytokine activation is a critical mechanism in the progression of both CHF and CKD. Markers of inflammation are predictive of all-cause mortality and cardiac death in patients with CKD. Pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and IL-6 activate the p38 and ERK1/2 (p42/44) mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NFκB) pathways. These MAPK cascades are well documented to mediate adverse effects on cardiac remodelling and heart failure. Nuclear factor-kappa B is involved in MAPK-mediated cardiac remodelling downstream of p38 and p42/44 MAPKs. Pro-inflammatory cytokines such as TNFα and IL-1β stimulate cardiac remodelling including hypertrophy and collagen synthesis (fibrosis). Thus, we hypothesized that IS may activate pro-inflammatory pathways in circulating immune cells such as monocytes to produce inflammatory cytokines that adversely affect cardiac function.

Based on the above considerations, we studied the potential pro-fibrotic, pro-hypertrophic, and pro-inflammatory effects of IS in cell culture.

Methods
We used isolated neonatal rat cardiac myocytes (NCMs) and fibroblasts (NCFs) as well as human leukaemia monocytic cell line (THP-1) cells in our study to investigate the hypothesis of interest: (i) IS, increased in CKD, may have pro-fibrotic and pro-hypertrophic effects on cardiac cells, which may adversely contribute to cardiac remodelling; and (ii) IS may activate pro-inflammatory pathways in circulating immune cells such as monocytes to produce inflammatory cytokines that adversely affect cardiac function.

Neonatal rat cardiac myocyte and fibroblast culture
Neonatal Sprague–Dawley NCMs and NCFs were isolated from 1- to 2-day-old pups with enzymatic digestion as described in detail previously. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (PHS Approved Animal Welfare Assurance no. A5587-01). All animal usage was also approved by St Vincent’s Hospital’s Animal Ethics Committee (AEC) in accordance with National Health and Medical Research Council (NHMRC) guide for the care and use of laboratory animals (AEC no. 42/06). Neonatal rat cardiac fibroblasts were seeded and maintained in high-glucose (25 mM) DMEM containing 5.33 mM KCl (Invitrogen, Mount Waverley, VIC, Australia) in the presence of 1% antibiotic/antimycotic (Invitrogen) and 10% FBS (JRH biosciences). Neonatal rat cardiac fibroblasts were used at passage 2. Purified NCMs were seeded (1000 cells/mm²) in 12-well plates and maintained in serum-free DMEM (Invitrogen) supplemented with insulin and transferring as described previously. Bromodeoxyuridine was included for the first 3 days. KCl (50 mM) was added to the medium to prevent contact-induced spontaneous contraction hypertrophy of the plated NCMs.

Measurement of neonatal rat cardiac myocyte hypertrophy
Rat cardiac myocyte hypertrophy was determined by 3H-leucine incorporation as previously described. Briefly, 2 h after pre-treatment with or without p38 MAPK inhibitor, RWJ-67657 (kind gift of Scott Wadsworth, PhD, Johnson & Johnson Pharmaceutical Research & Development, L.L.C.) (0.2–25 μM), or p42/44 MAPK inhibitor, U0126 (Sigma, St Louis, MO, USA) (0.03–10 μM), IS (Sigma) at a concentration range from 0.1 nM to 300 μM was added to cells in serum-free DMEM supplemented with insulin transferrin and 50 mM KCl and then incubated for a further 48 h before harvest. To each well, 1 μCi of 3H-leucine was also added. Cells were harvested by precipitation with 10% TCA on ice for 30 min before solubilization with 1 M NaOH overnight at 4°C. The samples were then neutralized with 1 M HCl, and 3H levels were counted in scintillation fluid on a beta counter to determine the levels of 3H-leucine incorporation.

Measurement of neonatal rat cardiac fibroblast collagen synthesis
Neonatal rat cardiac fibroblast collagen synthesis was determined by 3H-proline incorporation as described previously. Neonatal rat cardiac fibroblasts were seeded at a density of 50,000 cells/well in 12-well plates and incubated (5% CO₂) overnight before serum-starved with 0.5% bovine serum albumin (BSA) for 48 h. Cells were then pre-treated in the presence or absence of RWJ-67657 (1–25 μM) or U0126 (0.1–10 μM) for 2 h before stimulation with IS (0.1 nM to 200 μM) in the presence of 0.5% BSA and addition of 1 μCi of 3H-proline to each well. After 48 h of further incubation, cells were harvested by TCA precipitation, and 3H-proline incorporation was determined similar to cardiac myocyte hypertrophy assays described above.

Quantitative measurement of pro-inflammatory cytokine mRNA expression in THP-1 cells
After 48 h serum-starving in RPMI containing 5.33 mM KCl (Invitrogen) in the presence of 0.5% BSA, THP-1 cells were treated with IS (0.1–100 μM) for an 18 h incubation. Cells were then harvested
and total RNA was extracted using Qiagen RNeasy kit (Qiagen, Hilden, Germany). After being reverse-transcribed to cDNA with MultiScribe (Applied Biosystems, Foster City, CA, USA), triplicate cDNA aliquots were amplified using sequence-specific primers (Geneworks, Adelaide, SA, Australia) and TaqMan fluorogenic probe (Applied Biosystems) using an ABI prism 7900HT sequence Detection System (Applied Biosystems). Real-time polymerase chain reaction (PCR) was used to quantify pro-inflammatory cytokine gene expression (TNF-α, IL-6, and IL-1β). The primer pairs and probes were designed using Primer Express 2.0 software (Applied Biosystems) based on published sequences (http://www.ncbi.nlm.nih.gov) as previously described.31 18S rRNA was used as an endogenous control in all experiments to correct for the expression of each gene.

Measurement of cell viability in neonatal rat cardiac fibroblasts and THP-1 cells

Neonatal rat cardiac fibroblasts were seeded in 96-well plates at a density of 10,000 cells/well. After serum-starving for 48 h, NCFs were treated with IS (0.01–100 μM) in the presence or absence of RWJ-67657 or U0126. THP-1 cells were seeded in 96-well plates at a density of 20,000 cells/well after serum-starving for 48 h, and then treated with IS (0.01–100 μM) in the presence or absence of RWJ-67657 or U0126. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine proliferation and cell viability of NCFs and THP-1 cells as previously described.32

Western blot analysis

Neonatal rat cardiac fibroblast (1 × 10⁵ cells/flask) and NCMs (2 × 10⁵ cells/well) were plated in T-75 flasks and 6-well plates, respectively, and then serum-starved for 48 h. THP-1 cells were seeded in T-25 flasks at a density of 3 × 10⁵ cells/flask after serum-starving for 48 h. For all cell types, cells were pre-treated with or without RWJ-67657 or U0126 for 2 h prior to IS (0.01–100 μM for NCF and 10–100 μM for THP-1 cells) stimulation for 15 min. Cells were harvested and lysed with modified RIPA buffer in the presence of protease and phosphatase inhibitors. After centrifuged for 15 min, the cleared cell lysates were collected. Protein concentrations were measured by the Bradford assay (Bio-rad, Hercules, CA, USA). Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes (Amer sham Biosciences). Western blot analysis was performed as per manufacturer protocol with specific antibodies (Cell Signaling Technology, Beverly, MA, USA) and then visualized by enhanced chemiluminescent reagents (Thermo Scientific). Band intensity was analysed using program ImageJ software (NCBI).

Materials

Indoxyl sulfate and U0126 were purchased from Sigma. U0126 and RWJ were prepared with DMSO and the stock solution kept at −20°C until used. Indoxyl sulfate stock solution was prepared with endotoxin-free sterilized phosphate-buffered saline and kept at −20°C until used. Both DMEM and RPMI, containing 5.33 mM KCl according to the formulation provided by the manufacturer, were purchased from Invitrogen. Other reagents are as described above or of the highest grade available commercially.

Endotoxin assay

To eliminate the possibility of endotoxin contamination as a confounder, endotoxin concentrations in IS-containing media were measured by the limulus amebocyte lysate method using the ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA). Assays were performed according to the manufacturer’s suggested protocol.

Statistical analysis

Absolute values from each triplicate experiment for each condition were collected. Percentages were calculated by taking the mean of triplicate absolute values and comparing as a per cent change to the mean of triplicates of un-treated control absolute values within each individual experiment. The mean of these changes were then taken as average change (± SEM). Data are presented as means ± SEM. Experiments were performed at least three times in triplicate. One-way ANOVA with Neuman–Keul post hoc test was used for comparison among all groups and paired t-test was used for comparisons between groups using GraphPad Prism software version 5. Paired t-test was chosen as the appropriate statistical comparison because all the cells used in each experiment came from the same batch of cells pooled from many animals. Thus, the cell populations in each well were ‘identical’ among control and treated groups. A two-tailed P-value of less than 0.05 was considered statistically significant.

Results

Indoxyl sulfate directly stimulates cardiac fibroblast collagen synthesis

Treatment of cardiac fibroblasts occurred at IS concentrations ranging from 0.1 nM to 200 μM. Indoxyl sulfate 3 μM (the lowest effective dose) to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation (Figure 1A). This stimulation was up to 145.7% that of control (P = 0.0174).

The p38 MAPK inhibitor, RWJ-67657, and the MEK1/2 (upstream of ERK1/2) inhibitor, U0126, dose-dependently suppressed IS-stimulated collagen synthesis, indicating that IS stimulates NCF collagen synthesis at least in part via the p38 and p42/44 MAPK pathways (Figure 3A). Thus, treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation. Treatment of cardiac fibroblasts occurred at IS concentrations from 0.01 nM to 200 μM significantly stimulated NCF collagen synthesis, as determined by 3H-proline incorporation.
Indoxyl sulfate activates mitogen-activated protein kinase and nuclear factor-kappa B pathways directly in cardiac cells

Western blot analysis demonstrated that IS exerted its effect on cardiac cells via activation of MAPK and NFκB pathways. After 15 min of incubation, IS stimulated p38 MAPK, p42/44 MAPKs, and NFκB phosphorylation but did not affect JNK phosphorylation in NCF (Figure 4A). Furthermore, activation of p38 MAPK was inhibited by RWJ-67657 (Figure 4A). In NCM, IS stimulation significantly increased p42/44 MAPK and NFκB phosphorylation levels and to a lesser degree p38 MAPK (not significant) (Figure 4B). Thus, IS directly affects cardiac cells via activation of MAPK and NFκB pathways.
Figure 3 The effects of indoxyl sulfate on neonatal cardiac myocyte hypertrophy. (A) $^{3}$H-leucine incorporation analysis demonstrated that indoxyl sulfate stimulated rat cardiac myocyte hypertrophy significantly after 48 h of incubation. Angiotensin II (100 nM) has been included as positive control, and it is worth noting that IS have similar effects as AngII even at low concentrations. (B) Under the same conditions, the stimulating effect of IS on rat cardiac myocyte hypertrophy was inhibited by RWJ-67657 and U0126 dose-dependently, indicating that the effects may be at least in part via p38 and MEK1/2 pathways. Data presented as means ± SEM from five experiments with triplicates. ***P < 0.001 vs. control, **P < 0.01 vs. control, *P < 0.05 vs. control. ###P < 0.001 vs. 10 mM indoxyl sulfate, ##P < 0.01 vs. 10 mM indoxyl sulfate, #P < 0.05 vs. 10 μM indoxyl sulfate.

Figure 4 Western Blot analyses on signalling pathways activated by indoxyl sulfate treatment in cardiac cells. Western blot analyses have been performed with specific anti-phospho-protein antibodies from Cell Signalling Technology. Equal amount of protein (30 μg each lane) from cleared cell lysates after 15 min stimulation with indoxyl sulfate was separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (A) Indoxyl sulfate treatment, at a range of dosages, stimulates p38 MAPK, p42/44 MAPK and nuclear factor-kappa B pathways by increasing their phosphorylation level in neonatal rat cardiac fibroblasts. Indoxyl sulfate did not stimulate JNK pathways in neonatal rat cardiac fibroblasts as its phosphorylation level did not increase by indoxyl sulfate even at 100 μM. Pre-treated neonatal rat cardiac fibroblasts with RWJ-67657 (25 μM) inhibited the activation of p38 MAPK by indoxyl sulfate. Representative western blot is presented. (B) Similarly, 10 μM indoxyl sulfate activated p42/44 MAPK and nuclear factor-kappa B pathways in rat cardiac myocytes by significantly increasing their phosphorylation levels as determined by western blot analysis. To a lesser degree, 10 μM indoxyl sulfate also activated the p38 MAPK pathway, although the increase in its phosphorylation levels did not quite reach significance. Representative western blot is presented with each condition in triplicate. The semi-quantitative score of each band is analysed with ImageJ, and data are presented as means ± SEM (n = 3). *P < 0.05 vs. control.
Indoxyl sulfate stimulates pro-inflammatory cytokine mRNA expression in THP-1 cells via mitogen-activated protein kinase and nuclear factor-kappa B pathways

Real-time PCR analysis from total RNA extracted from THP-1 cells after 18 h IS treatment showed that IS significantly stimulated TNF-α, IL-6, and IL-1β mRNA expression (Figure 5). Indoxyl sulfate stimulated TNF-α, IL-6, and IL-1β mRNA, and the expression was suppressed by RWJ-67657 and U0126 (data not shown).

Western blot analysis demonstrated that IS activated p38 MAPK, p42/44 MAPK, and NF-κB pathways by increasing their phosphorylation levels after 15 min of incubation in THP-1 cells, peaking at a concentration of 10 μM (Figure 6A–C, E and F). Interestingly, IS did not stimulate JNK pathway in THP-1 cells (Figure 6G) concordant with its (lack of) effect in cardiac cells (Figure 4A). Furthermore, western Blot analysis also showed that the activation of p38 and p42/44 MAPKs by IS was inhibited by RWJ-67657 and U0126, respectively (Figure 6B and C). Activation of inflammatory-related transcription factor NFκB by IS was also suppressed by both RWJ-67657 and U0126 (Figure 6E and F). Indoxyl sulfate treatment did not affect the total protein level of p42/44 MAPK under the same experimental condition, with or without U0126 treatment (Figure 6D).

Results from MTT assays showed that IS co-culture at a dose of up to 100 μM did not affect THP-1 cell proliferation and viability (Figure 7). Additionally, the presence of RWJ-67657 or U0126 did not affect THP-1 cell viability as determined by the MTT assay (Figure 7).

Endotoxin assay

The endotoxin levels of IS solutions were below the lowest standard, and detection limit (0.005 EU/mL) at all IS concentrations was tested (up to 500 μM) (data not shown). These findings strongly indicate that the above effects observed in the present study were not due to endotoxin contamination.

Discussion

The present study has found, for the first time, that a uraemic toxin, IS, may directly mediate a number of potentially important detrimental effects on cardiac cellular function. In particular, we have observed in cell culture direct pro-fibrotic, pro-hypertrophic, and pro-inflammatory effects of this toxin. These findings have considerable implications for cardiac remodelling in the setting of heart failure and co-morbid CKD, where levels of this toxin are elevated.

Among a variety of uraemic toxins, IS was of particular interest because it is inadequately dialyzable and levels are markedly elevated in the CKD setting. Whether co-morbid CHF further contributes to additional elevation in plasma levels is uncertain as it has not as yet been measured in this setting. In addition, IS has been found to be pro-fibrotic in renal mesangial cells, and chronic infusion of IS has been found to result in adverse functional effects on the kidney.10

Neonatal cardiac cells were used in this study as they have proved to be a valid model of cardiac myocyte hypertrophy and fibrosis as well as for evaluating the efficacy of therapeutic agents.28,30 Neonatal cardiac myocytes also express early genes (such as ANP) and exert the same phenotype of cardiac hypertrophy of adult cells.30,34

Given that chronic cardiac dysfunction may also be contributed to by pathological fibrosis, we sought to determine whether IS may be similarly pro-fibrotic in the heart. We found an increase in collagen synthesis (as assessed by 3H-proline incorporation) in NCF cell culture, which was observed even at low concentrations down to 3 μM, noting that circulating levels of IS in CKD patients varies from few micromolars to hundreds of micromolars.5,6

Another major mechanism of cardiac remodelling involves hypertrophy of myocytes. In a manner analogous to the studies in cardiac fibroblasts, cardiac myocytes cultured in the presence
of IS resulted in significant myocyte hypertrophy, which was again inhibited via the p38 and p42/44 MAPK pathways.

As aforementioned, circulating IS can be found in healthy subjects (at very low levels), but the free form is essentially non-detectable. This suggests that the equilibrium state of IS protein-binding capacity may differ between CKD patients and normal subjects. It is therefore unlikely that IS exerts any putative adverse cardiac effects until renal excretory function is significantly impaired.

Figure 6  Western blot analyses on signalling pathways activated by indoxyl sulfate treatment in THP-1 cells. Western blot analyses have been performed with specific anti-phospho-protein antibodies from Cell Signalling Technology. Equal amount of protein (30 μg each lane) from cleared cell lysates after 15 min stimulation with indoxyl sulfate was separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (A) Indoxyl sulfate treatment, at a range of dosages, stimulates p38 MAPK, p42/44 MAPK, and nuclear factor-kappa B pathways by increasing their phosphorylation level in THP-1 cells. In this representative, western blot showed that 10 μM indoxyl sulfate activated p38 MAPK, p42/44 MAPK, and nuclear factor-kappa B signalling pathways most effectively after 15 min of incubation. Western blot analysis with samples from 10 μM indoxyl sulfate treatment for 15 min in triplicate demonstrated indoxyl sulfate increased the phosphorylation levels of p38 MAPK (B), p42/p44 MAPK (C), and nuclear factor-kappa B (E, F) significantly. Indoxyl sulfate did stimulate JNK phosphorylation under the same conditions (G). In the presence of 1 μM RWJ-67657, the increase in the phosphorylation levels of both p38 MAPK (B) and nuclear factor-kappa B (E) was abolished. Similarly, 1 μM U0126 abolished the increase in phosphorylation levels of p42/44 and NFκB (C, F) increased by indoxyl sulfate. Indoxyl sulfate treatment did not affect the levels of total p42/p44 MAPK protein even in the presence of 1 μM U0126 (D), indicating that indoxyl sulfate did not affect protein phosphorylation by changing the protein levels. The semi-quantitative score of each band is analysed with ImageJ, and data are presented as means ± SEM (n = 3). **P < 0.01 vs. control, *P < 0.05 vs. control, ##P < 0.01 vs. 10 μM indoxyl sulfate, ###P < 0.05 vs. 10 μM indoxyl sulfate.

Median plasma/serum uraemic IS levels have been reported in eight individual studies from five different research groups across a wide range (40 up to ~500 μM) in subjects with advanced renal dysfunction and/or glomerular filtration rate ≤ 30 mL/min/1.73 m² (stages 4 and 5 CKD). Recently, serum IS was reported to be significantly increased in stage 3 CKD in type 2 diabetic nephropathy at a mean level of 5.18 μM, comparable to the lowest concentration causing increased collagen synthesis in this study. Left ventricular hypertrophy, commonly associated with
cardiac fibrosis, can be detected as early as stage 3 in patients with CKD. The prevalence of LVH combining stages 3 and 4 is 25–50%, which rises to ~80% at the time of first dialysis (beginning of stage 5 CKD). This occurs quite early in the evolution of CKD and may only be partially explained by co-morbid hypertension, volume overload, or low haemoglobin levels. Cardiac structural changes are documented to be in part independent of high blood pressure. Thus, it is possible that IS may accelerate pathological cardiac hypertrophy with accompanying fibrosis, independent of the above co-morbid drivers from the early stages of CKD. In summary, based on the above observations, we have noted in the present study both increased cardiac collagen turnover and myocyte hypertrophy at in vitro concentrations of IS that fall approximately into this clinical patho-physiological concentration range (additionally taking into account protein-binding characteristics of the toxin).

The present study also attempted to delineate pathways by which this pro-fibrotic and pro-hypertrophic action may have occurred within cardiac fibroblasts and myocytes. Activation of the p38 and p42/44 MAPK pathways as well as the NFκB pathway was observed with IS. Inhibition of p38 MAPK with RWJ-67657 and p42/44 MAPK with U0126 (MEK1/2, upstream of p42/44 MAPK, inhibitor) abrogated IS-activated signalling pathways as well as collagen synthesis and hypertrophic effects in cardiac cells. Thus, IS appears to exert at least some of its effects on cardiac cells via activation of p38 and p42/p44 MAPK and NFκB pathways, which are also important in inflammatory responses.

The effects of IS on immune cells have also been evaluated in this study using a monocytic cell line (THP-1 cells). Indoxyl sulfate treatment with these cells resulted in increased expression of TNF-α, IL-6, and IL-1β genes. These findings are of particular relevance to both fibrosis and hypertrophy because pro-inflammatory cytokine activation is stimulatory to both of these processes. Thus, in addition to its direct effects on cardiac cells, IS enhances inflammatory cytokine production from circulating monocytic cells, which can further exacerbate pathological cardiac remodelling, including hypertrophy and fibrosis. Furthermore, increased inflammatory cytokine production by IS can contribute to damage in other organs, including the kidney and the heart, via such processes.

It is important to note that all of the above observations occurred in the absence of any significant reduction in cell viability, as assessed by the MTT assay. Furthermore, there was no detectable endotoxin in the IS used in the present study (as measured by the LAL Endotoxin Assay Kit), indicating the cardiac cellular effects observed were clearly not due to endotoxin contamination.

Notwithstanding the above dosing considerations, there may be considerable clinical implications to the above findings. The so-called cardiorenal syndrome describes the complex inter-relationship between the heart and the kidney in the setting of impaired cardiac function, kidney hypoperfusion, and activation of various circulating factors from the kidney feeding back to further reduce cardiac function. While the prevailing thinking is that these renal factors comprise primarily the activation of neurohormonal processes such as the renin–angiotensin–aldosterone system, the findings of the present study suggest a new potential link between the kidney and the heart, that being non-haemodialyzable uraemic toxins contributing to chronic cardiac dysfunction and remodelling.

Indoxyl sulfate is the product of diet-derived tryptophan being converted by intestinal flora to indole and finally IS in the body. Thus, inhibition of production of IS or its precursors in the gut may represent a potential novel therapeutic approach to the treatment of the cardiorenal syndrome and heart failure in general. Adsorptive agents such as AST-120 (Kremezin) may be of therapeutic value in this regard. Studies with this agent have demonstrated beneficial renal effects in CKD. We therefore propose that studies with Kremezin in CHF with co-morbid CKD may be of considerable therapeutic interest, additional to conventional heart failure therapies.
In summary, we have observed that a uraemic toxin, IS, may contribute to direct adverse cardiac effects in cell culture, and do so at a clinically relevant concentration range. Indoxyl sulfate (and potentially other uraemic toxins) may therefore represent an important novel therapeutic target for the amelioration of the cardio-renal syndrome in co-morbid CKD and heart failure.

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