The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review

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ABSTRACT

A growing number of publications supports a biologic effect of the protein-bound uremic retention solutes indoxyl sulfate and p-cresyl sulfate. However, the use of unrealistically high free concentrations of these compounds and/or inappropriately low albumin concentrations may blur the interpretation of these results. Here, we performed a systematic review, selecting only studies in which, depending on the albumin concentration, real or extrapolated free concentrations of indoxyl sulfate and p-cresyl sulfate remained in the uremic range. The 27 studies retrieved comprised in vitro and animal studies. A quality score was developed, giving 1 point for each of the following criteria: six or more experiments, confirmation by more than one experimental approach, neutralization of the biologic effect by counteractive reagents or antibodies, use of a real-life model, and use of dose-response analyses in vitro and/or animal studies. The overall average score was 3 of 5 points, with five studies scoring 5 of 5 points and six studies scoring 4 of 5 points, highlighting the superior quality of a substantial number of the retrieved studies. In the 11 highest scoring studies, most functional deteriorations were related to uremic cardiovascular disease and kidney damage. We conclude that our systematic approach allowed the retrieval of methodologically correct studies unbiased by erroneous conditions related to albumin binding. Our data seem to confirm the toxicity of indoxyl sulfate and p-cresyl sulfate and support their roles in vascular and renal disease progression.


As CKD evolves, uremic retention becomes a progressively more important contributor to overall organ dysfunction.1–3 Among the three physicochemical types of uremic solutes,4 the pathophysiologic importance of protein-bound toxins has been neglected for a long time,5–7 leading to a shortage of specific removal strategies.8,9 In the past decade, however, a growing number of publications documented the impact of protein-bound uremic toxins on vital processes and an association of their concentration with clinical outcome parameters.6,10–19

One factor blurring the interpretation of the biochemical effects of uremic toxins is the application of unrealistic concentrations compared with the concentrations observed in human CKD in in vitro testing or in vivo animal experiments.5,20 Moreover, for protein-bound toxins, even with seemingly acceptable total concentrations, the quantities of albumin or protein present are often too low, resulting in unacceptably high and thus, irrelevant free concentrations.

In analogy to drugs, albumin is likely to act as a buffer, attenuating potential biochemical effects of its ligands,21–23 whereas recent data clearly showed that, for both indoxyl sulfate and p-cresyl sulfate, albumin is the main binding protein.24 Absence of appropriate albumin quantities results in too high free solute concentrations, inducing exaggerated biologic responses with a potential for an overestimated toxic impact. This bias has provoked justified objections against recognizing protein-bound molecules as real uremic toxins. In a recent editorial,25 indoxyl sulfate, one of the main protein-bound solutes, was for that reason called “long suspected but not yet guilty.”

Nevertheless, a careful check of the literature reveals that investigators in a number of studies have applied acceptable free concentrations by administering those toxins to animals up to acceptable total concentrations, adding relevant quantities of albumin to in vitro media by using whole blood, plasma, or serum, or in the absence of albumin, applying concentrations corresponding to the free uremic fraction. At this moment, it is unclear, however, how many methodologically suitable publications are available and what they teach us about the contribution of the compounds to the uremic syndrome.

In the present analysis, we applied a systematic search to filter out those studies in which unacceptably high free concentration of protein-bound solutes had been applied. We aimed to analyze the effect of indoxyl sulfate and p-cresyl sulfate in vitro and in animals. Based on
present standards, 27 articles of adequate quality were retrieved and are reported here together with the shown effects.

RESULTS

The literature was searched according to preset methods (see below) to retrieve studies on biologically relevant toxic effects of indoxyl sulfate and p-cresyl sulfate following well defined quality standards. One of the conditions was that the concentrations used in those studies conformed with the concentrations observed in uremia, which is illustrated in Table 1. The concentrations considered appropriate were in the high uremic range to compensate for the often short exposure time in the experimental studies, especially in vitro. To make a comparison with the values currently observed in an average dialysis population, we give in Table 1, also as reference, data retrieved from a recent study from the European Uremic Toxin Work Group as a real-life orientation for the reader.20 We identified 336 citations through electronic searching and added 61 citations from reference lists of retrieved publications (Figure 1). Based on the selection criteria, we included 27 studies in the review (Table 2).26–52

The number of retrievable studies increased considerably over the past years, suggesting a rising awareness about the topic. The distribution among countries points to a broad worldwide interest. The majority (n=20) was generated in Asia, especially Japan; however, seven studies in total were performed in Europe, the United States, or Australia.

The following cell and organ systems were investigated (n of each study is in parentheses) (Table 2): renal tubules (9),34,36–39,43,45,47,50 endothelium (6),26–30,33 leukocytes (2),30,31 whole vessels (2),40,41 whole kidney (2),40,42 cardiac cells (2),35,49 smooth muscle cells (2),46,51 hepatocytes (2),32,51 intestinal cells (1),48 and adipocytes (1).44 A few studies concentrated on more than one target system: one study was on both the whole vessel and kidney40 and one study was on leukocyte–endothelial interaction.30

Twenty-four studies concentrated on indoxyl sulfate,26–30,32–43,46–52 and six studies concentrated on p-cresyl sulfate.31,34,36,39,44,45 Three studies evaluated both compounds.34,36,39 Some studies also assessed alterations induced by other uremic toxins, but only two of those studies showed a significant effect for indole acetic acid,46,48 hippuric acid,48 and carboxyl-methyl-propyl-furanpropionic acid.48

All studies evaluating indoxyl sulfate showed a negative (toxic) effect (Table 3), except the study by Odamaki et al.,52 which showed an increase of hepatocyte albumin production in the presence of indoxyl sulfate. Four studies on p-cresyl sulfate53–56 and one study on indoxyl sulfate55 showed no significant changes (data not shown).

Of 14 animal studies, 10 studies were in rats,37,38,40–43,45,47,49,50 and four studies were in mice.30,36,39,44 Several of the rat studies were in the specific Dahl salt-sensitive hypertensive rat strain,37,38,40–43,47,49,50 although in some of these studies, an effect in the wild type was also observed.37,38,43,47,50 One study only evaluated Dahl wild-type rats and found a significant effect of indoxyl sulfate in that strain.42

Of the in vitro studies, eight studies applied an appropriate concentration of albumin, resulting in relevant free toxin concentrations,26–29,31,46,51,52 whereas in the remaining seven studies, a concentration compatible with the uremic free fraction was pursued.32–36,39,48

A large array of mediators, pathways, and messenger molecules contributed to the observed system effects (Table 4). Several were confirmed in more than one study of this series.27,30,34–36,38,40,43,45,48–50 Several of the observed effects were related to inflammation/free radical production27,30,34–36,44,45,48–50 and fibrosis,36,38–40,43,49 The mutual connections of these factors into global pathways for endothelial and proximal tubular cells are shown in Figure 2.

With regards to quality, all retained studies together had a median score of 3.0 from a possible total of 5 points, indicating that approximately one half of the retained studies had a score of 3 points or above (Supplemental Material 1). Five studies reached a score of 5 of 5 points,35,36,39,44,45 and six studies obtained a score of 4 of 5 points.27,30,38,46,49,50 These 11 studies with a high quality score covered a broad array of pathophysiologic mechanisms mainly related to cardiovascular damage and progression of kidney disease: reactive oxygen species generation (3),27,45,50 endothelial dysfunction (2),27,30 epithelial-to-mesenchymal transition (2),36,38 leukocyte–endothelial interaction (1),30 deterioration of cardiac cell functional capacity (1),35 Klotho expression (1),39 expression of tissue factor in vascular smooth muscle cells (1),46 and insulin resistance (1).44 The reasons for scoring 4 of 5 points instead of 5 of 5 points were lack of proof of concept by effect neutralization two times, the number of experiments was too low two times, lack of confirmation by an alternative approach one time, and absence of real-life conditions one time. There was no significant

Table 1. Threshold values of concentrations

<table>
<thead>
<tr>
<th></th>
<th>Normal (mg/L)</th>
<th>Uremia (mg/L)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS (molecular weight 212)</td>
<td></td>
<td>236.04</td>
<td>Highest individual value</td>
</tr>
<tr>
<td>ISfree high</td>
<td>0.530</td>
<td>4.520</td>
<td>Highest mean</td>
</tr>
<tr>
<td>PCS (molecular weight 188)</td>
<td></td>
<td>105.078</td>
<td>Highest individual value</td>
</tr>
<tr>
<td>PCSfree high</td>
<td>0.12</td>
<td>2.620</td>
<td>Highest mean</td>
</tr>
</tbody>
</table>

Highest individual value is the highest single value per patient ever reported; highest mean is the highest mean for a patient group ever reported. Current average values observed in an average dialysis population as reported in the most recent review of the European Uremic Toxin Group are 23.1 mg/L for IStotal and 20.9 mg/L for PCStotal.20 IS, indoxyl sulfate; LOD, limit of detection; PCS, p-cresyl sulfate.
correlation between year of publication and quality score (data not shown).

DISCUSSION

This study reviews the literature evidence up until June 30, 2013, for the biologic and/or toxic effects of two prototype protein-bound uremic toxins: indoxyl sulfate and p-cresyl sulfate. In total, 27 studies from all over the world were retained, with the principal topics being endothelial and proximal tubular cell dysfunction, although insulin resistance, functional status of cardiac cells, leukocyte dysfunction, coagulation disturbances, and pharmacodynamics were also covered. A quality assessment identified 11 of 27 (41%) studies with a score of at least 4 points from a possible maximum of 5 points. The majority of the changes found affects mechanisms that are essential to the pathophysiology of the uremic syndrome and its subsequent detrimental impact on prognosis.

Endothelial dysfunction,26–30,33 smooth muscle cell lesions,46,51 coagulation disturbances,46 leukocyte activation,30,31 cross-talk of leukocytes and endothelium,30 cardiac fibrosis and hypertrophy,35,49 and insulin resistance48 as well as whole-vessel alterations per se40,41 are all linked to cardiovascular damage and contribute to the increased propensity for mortality and cardiovascular events in CKD.57,58 Both indoxyl sulfate and p-cresyl sulfate per se have repeatedly been related to cardiovascular mortality.10–19 Although strategies that more efficiently remove protein-bound solutes, such as frequent dialysis59 and online hemodiafiltration,60,61 in controlled studies are associated with improved outcomes,62,63 it remains difficult to interpret these data because of the presence of confounding factors that affect the outcomes on their own and a lack of selectivity of removal, because dialysis strategies are usually not restricted to protein-bound solutes alone.

Next to the above cardiovascular problems, the data linking these solutes to tubular cell damage,39,45 tubular epithelial-to-mesenchymal transition,36,38 tubulointerstitial inflammation and fibrosis,34,36,37,39,47,50 or whole-kidney damage40,42 are all linked to the progression of CKD, another factor related to uremic morbidity and mortality.57 As a consequence, decrease in concentration of intestinally generated uremic re- tention solutes, like indole, by the intestinal sorbent AST-120 (Kremezin) has been accepted in mostly Asian countries as a strategy to restrain the progression of CKD. It is very likely that this sorbent affects the concentration of not only indoxyl sulfate but also, other protein-bound uremic toxins, one of which is p-cresyl sulfate.64 In at least two small-sized randomized controlled trials, AST-120 had a positive effect on progression of CKD.63,66 In a recent large randomized controlled trial in the United States and Europe, however, this positive effect could not be confirmed.67

Until now, biologic data were perceived as not consistent or convincing enough to recognize protein-bound toxins as the real culprits.25,68,69 The results of the current analysis applying systematic search methods linked to a quality assessment of the retrieved publications, however, yielded several high-quality papers applying, for the uremic setting, acceptable concentrations and state-of-the-art test methods. Therefore, our analysis offers, in our opinion, arguments that are solid enough to reconsider the reluctance to recognize the protein-bound solutes indoxyl sulfate and p-cresyl sulfate as toxic.

This study is, to the best of our knowledge, the first to apply to biologic studies a stringent strategy that had been defined in advance, in casu related to uremic retention, resulting in a systematized summary of their toxicity. Although we tried to follow current standards for the conduct of systematic reviews,70 there are methodological deviations. In contrast to evidence-based systematic reviews, in our case, only a limited number of search engines was used. Of note, for biologic studies, especially in the area of uremia, there is, to the best of our knowledge, not a large repository of data such as the one made available by the Cochrane Library (Cochrane Central Register of Controlled Trials) for clinical studies. Our approach may serve

Figure 1. Flowchart of the review procedure and quality scoring.
as a basis for future analyses of the same kind.

If we consider a quality score of 4 or 5 points of 5 points as the equivalent of a high evidence label, the results reported in the present article should provide a positive incentive to developing strategies to remove these protein-bound molecules better and more consistently than at present. Alternatives to the standard approaches that we now know to have a potentially beneficial impact are extended dialysis,71 prebiotics,72,73 probiotics72,73 and extracorporeal sorbents.74 Because our knowledge regarding this issue is extending, alternative options may arise in the nearby future. Anyway, the fact that dietary components and residual renal function seem to affect protein-bound solute concentration more than dialysis adequacy, as assessed by Kt/V,75 may suggest that alternative methods focusing on preserving kidney function or affecting intestinal toxin generation

<table>
<thead>
<tr>
<th>First Author, Year, and Reference</th>
<th>Cell/Organ System</th>
<th>Toxin</th>
<th>Concentration (mg/L)/Dose</th>
<th>Albumin/Type Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) In vitro, albumin: normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dou, 200426</td>
<td>Endothelium</td>
<td>IS</td>
<td>25–250</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Odamaki, 200452</td>
<td>Hepatocytes</td>
<td>IS</td>
<td>50–100</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Faure, 200628</td>
<td>Endothelium</td>
<td>IS</td>
<td>256</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Yamamoto, 200651</td>
<td>Smooth muscle cells</td>
<td>IS</td>
<td>53–106</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Dou, 200727</td>
<td>Endothelium</td>
<td>IS</td>
<td>125–250</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Schepers, 200731</td>
<td>Leukocytes</td>
<td>PCS</td>
<td>121.0</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Itoh, 201229</td>
<td>Endothelium</td>
<td>IS</td>
<td>29.9–57.2</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Chitalia, 201346</td>
<td>Smooth muscle cells</td>
<td>IS</td>
<td>25</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Koppe, 201354</td>
<td>Adipocytes, myotubes</td>
<td>PCS</td>
<td>40</td>
<td>35 g/L</td>
</tr>
<tr>
<td>(2) In vitro, albumin: low, absent, or unspecified</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tsujimoto, 201032</td>
<td>Liver microsomes</td>
<td>IS</td>
<td>6.36</td>
<td>Absent</td>
</tr>
<tr>
<td>Lekawanvijit, 201035</td>
<td>Cardiac fibroblasts/myocytes</td>
<td>IS</td>
<td>0.02–63.6</td>
<td>5 g/L BSA/absent</td>
</tr>
<tr>
<td>Yu, 201133</td>
<td>Endothelium</td>
<td>IS</td>
<td>1.25–125</td>
<td>Unspecified</td>
</tr>
<tr>
<td>Sun, 201334</td>
<td>Proximal tubular cells</td>
<td>IS</td>
<td>1; 5</td>
<td>Absent</td>
</tr>
<tr>
<td>Sun, 201236</td>
<td>Proximal tubular cells</td>
<td>IS</td>
<td>1–50</td>
<td>Absent</td>
</tr>
<tr>
<td>Sun, 201239</td>
<td>Proximal tubular cells</td>
<td>IS</td>
<td>1; 5</td>
<td>Unspecified</td>
</tr>
<tr>
<td>Tsujimoto, 201248</td>
<td>Intestinal cells (hepatic no effect)</td>
<td>IS</td>
<td>4.24</td>
<td>0.8 g/L (10% FBS)</td>
</tr>
<tr>
<td>(3) Animala</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adijang, 200840</td>
<td>Aorta, kidney</td>
<td>IS</td>
<td>23.1/200 mg/kg (30 wk) p.o.</td>
<td>DH rat</td>
</tr>
<tr>
<td>Ito, 201030b</td>
<td>Endothelium/leukocyte interaction</td>
<td>IS</td>
<td>15.7/200 mg/kg q.d. (10 d) p.o.</td>
<td>Nx mouse</td>
</tr>
<tr>
<td>Adijang, 201041</td>
<td>Aorta</td>
<td>IS</td>
<td>15–20/200 mg/kg (32 wk) p.o.</td>
<td>DN, DH rat</td>
</tr>
<tr>
<td>Bolati, 201130b,c</td>
<td>Tubular cells</td>
<td>IS</td>
<td>9.4–18.9/200 mg/kg (32 wk) p.o.</td>
<td>DH rat</td>
</tr>
<tr>
<td>Sun, 201236</td>
<td>Tubular cells</td>
<td>IS</td>
<td>1/2 Nx mouse</td>
<td>1/2 Nx mouse</td>
</tr>
<tr>
<td>Watanabe, 201345b</td>
<td>Renal tubular cells</td>
<td>PCS</td>
<td>32.6/50 mg/kg q.d. (4 wk) i.p.</td>
<td>5/6 Nx rat</td>
</tr>
<tr>
<td>Sun, 201239</td>
<td>Proximal tubular cells</td>
<td>IS</td>
<td>8.5</td>
<td>1/2 Nx mouse</td>
</tr>
<tr>
<td>Shimizu, 201237b,c</td>
<td>Proximal tubular cells</td>
<td>IS</td>
<td>100 mg/kg q.d. (4 wk) i.p.</td>
<td></td>
</tr>
<tr>
<td>Shimizu, 201342b</td>
<td>Kidney (cortex)</td>
<td>IS</td>
<td>9.4–18.9/200 mg/kg (32 wk) p.o.</td>
<td>DN, DH rat</td>
</tr>
<tr>
<td>Shimizu, 201343b</td>
<td>Tubular cells</td>
<td>IS</td>
<td>9.4/200 mg/kg q.d. (32 wk) p.o.</td>
<td>DN rat</td>
</tr>
<tr>
<td>Koppe, 201344</td>
<td>Adipocytes, myocytes, various organs</td>
<td>PCS</td>
<td>51/10 mg/kg b.i.d. (4 wk) i.p.</td>
<td>Normal RF mouse</td>
</tr>
<tr>
<td>Shimizu, 201347b,c</td>
<td>Tubular cells</td>
<td>IS</td>
<td>9.4–18.9/200 mg/kg (32 wk) p.o.</td>
<td>DN, DH rat</td>
</tr>
<tr>
<td>Yisireyiili, 201349</td>
<td>Cardiac tissue</td>
<td>IS</td>
<td>18.9/200 mg/kg q.d. (32 wk) p.o.</td>
<td>DN, DH rat</td>
</tr>
<tr>
<td>Bolati, 201350b</td>
<td>Tubular cells</td>
<td>IS</td>
<td>9.4/200 mg/kg q.d. (32 wk) p.o.</td>
<td>DN, DH rat</td>
</tr>
</tbody>
</table>

Is, indoxyl sulfate; PCS, p-cresyl sulfate; BSA, bovine serum albumin; FBS, fetal bovine serum; wk, weeks; p.o., per os; q.d., once a day; i.p., intraperitoneal; b.i.d., twice per day; DH, Dahl salt-sensitive hypertensive; Nx, nephrectomized; DN, Dahl salt-resistance normotensive; RF, renal function.

aFor animal experiments, we presumed that serum albumin was present at the same concentrations as usually in these animals; all solute concentrations were measured in serum, except for the study by Ito et al.30 (plasma), and steady state at the end of exposure on euthanasia of the animals, except for the study by Koppe et al.44 (peak after i.p. injection). Also, dose and administration route are mentioned; the Albumin/Type Model column contains both species and type of model.

bIn vitro part not appropriate.

cIf concentration is 9.4–18.9, it is 9.4 for Dahl wild-type rats and 18.9 for DH rats.
may be as important as extracorporeal removal.

The question could be raised as to how far toxic mechanisms and pathways overlap for both toxins. It is only possible if studies on the two compounds assess the same elements. To the best of our knowledge, for the time, such a parallel approach has only been used in renal tubular cells. Figure 2B confirms that, indeed, some mechanisms are overlapping.

This study has a few drawbacks. First, we focused only on two of the protein-bound solutes, whereas several other compounds in this group might have a biologic impact as well.\(^3\),\(^5\),\(^6\),\(^7\) We selected, however, the two molecules that have been most extensively evaluated. Some of the retained studies also show similar effects for other protein-bound solutes,\(^46\),\(^48\) suggesting that the yielded evidence could have been even more convincing if more compounds would have been taken into account. Second, we accepted concentrations up to the upper uremic range (Table 1),\(^4\),\(^20\),\(^77\),\(^78\) whereas in the average uremic patient, the concentration will be lower. However, a high concentration was applied essentially in vitro, where the concentration should be considered to compensate for short exposure to uremic toxins, in contrast to real life, where it is continuous, allowing solutes more time to reach the intracellular compartments where biologic activity is exerted. It is also of note that, in the in vivo animal studies, depending on the way of administration (intraperitoneally versus per os), the

<table>
<thead>
<tr>
<th>Cell/Organ System</th>
<th>Toxin</th>
<th>Main Observed Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Endothelium</td>
<td>IS</td>
<td>Inhibition proliferation and repair</td>
</tr>
<tr>
<td>Dou, 2004(^{26})</td>
<td>IS</td>
<td>Increase endothelial microparticle release</td>
</tr>
<tr>
<td>Faure, 2006(^{28})</td>
<td>IS</td>
<td>Induction ROS, inhibition glutathione</td>
</tr>
<tr>
<td>Dou, 2007(^{27})</td>
<td>IS</td>
<td>Increase endothelial/leukocyte interaction (adhesion)</td>
</tr>
<tr>
<td>Ito, 2010(^{30})</td>
<td>IS</td>
<td>Inhibition proliferation; increase senescence; increase ROS and decrease NO production</td>
</tr>
<tr>
<td>Yu, 2011(^{33})</td>
<td>IS</td>
<td>Induction ROS</td>
</tr>
<tr>
<td>Itoh, 2012(^{29})</td>
<td>IS</td>
<td>Increase proliferation</td>
</tr>
<tr>
<td>(2) Smooth muscle cells</td>
<td>IS</td>
<td>Generation tissue factor (linked to clot formation), tissue factor breakdown retarded</td>
</tr>
<tr>
<td>Yamamoto, 2006(^{51})</td>
<td>IS</td>
<td>Activation oxidative burst</td>
</tr>
<tr>
<td>Chitalia, 2013(^{46})</td>
<td>IS</td>
<td>Increase endothelial/leukocyte interaction (adhesion)</td>
</tr>
<tr>
<td>Schepers, 2007(^{31})</td>
<td>PCS</td>
<td>Increase aortic calcification and stiffness, expression osteoblast markers, and OATs</td>
</tr>
<tr>
<td>Ito, 2010(^{30})</td>
<td>IS</td>
<td>Induction cell senescence</td>
</tr>
<tr>
<td>(3) Leukocytes</td>
<td>IS</td>
<td>Cardiomyocyte proliferation, cardiac fibrosis, inflammation</td>
</tr>
<tr>
<td>Lekawawnvijit, 2010(^{35})</td>
<td>IS</td>
<td>Increase cardiomyocyte hypertrophy, cardiac fibrosis, oxidative stress</td>
</tr>
<tr>
<td>(4) Whole vessels</td>
<td>IS</td>
<td>Increase fibrosis</td>
</tr>
<tr>
<td>Adijang, 2008(^{40})</td>
<td>IS</td>
<td>Increase angiotsinogen expression</td>
</tr>
<tr>
<td>Adijang, 2010(^{41})</td>
<td>IS</td>
<td>Increase epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>Sun, 2013(^{34})</td>
<td>IS, PCS</td>
<td>Enhanced expression cytokine and inflammatory genes</td>
</tr>
<tr>
<td>Sun, 2012(^{26})</td>
<td>IS, PCS</td>
<td>Activation RAAS and epithelial-to-mesenchymal transition, fibrosis, nephrosclerosis</td>
</tr>
<tr>
<td>Shimizu, 2012(^{37})</td>
<td>IS</td>
<td>Increase Klotho gene methylation, renal fibrosis</td>
</tr>
<tr>
<td>Sun, 2012(^{29})</td>
<td>IS, PCS</td>
<td>Increase renal tubular damage</td>
</tr>
<tr>
<td>Watanabe, 2012(^{45})</td>
<td>PCS</td>
<td>Induction mediators for fibrosis (TGF-(\beta) and Smad3)</td>
</tr>
<tr>
<td>Shimizu, 2013(^{43})</td>
<td>IS</td>
<td>Induction generation adhesion molecule (ICAM-1)</td>
</tr>
<tr>
<td>Bolati, 2013(^{30})</td>
<td>IS</td>
<td>Increased oxidative stress</td>
</tr>
<tr>
<td>(5) Cardiac cells</td>
<td>IS</td>
<td>Increase fibrosis</td>
</tr>
<tr>
<td>Lekawawnvijit, 2010(^{35})</td>
<td>IS</td>
<td>Increase angiotsinogen expression</td>
</tr>
<tr>
<td>Itoh, 2012(^{29})</td>
<td>IS</td>
<td>Increase epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>Shimizu, 2013(^{37})</td>
<td>IS</td>
<td>Increase expression MCP-1</td>
</tr>
<tr>
<td>Sun, 2012(^{29})</td>
<td>IS, PCS</td>
<td>Increase renal tubular damage</td>
</tr>
<tr>
<td>Shimizu, 2013(^{43})</td>
<td>IS</td>
<td>Induction mediators for fibrosis (TGF-(\beta) and Smad3)</td>
</tr>
<tr>
<td>Bolati, 2013(^{30})</td>
<td>IS</td>
<td>Increased oxidative stress</td>
</tr>
<tr>
<td>(6) Whole heart</td>
<td>IS</td>
<td>Increase fibrosis</td>
</tr>
<tr>
<td>Yisireyiil, 2013(^{49})</td>
<td>IS</td>
<td>Increase angiotsinogen expression</td>
</tr>
<tr>
<td>(7) Renal tubular cells</td>
<td>IS</td>
<td>Increase fibrosis</td>
</tr>
<tr>
<td>Bolati, 2011(^{38})</td>
<td>IS</td>
<td>Increase epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>Sun, 2013(^{34})</td>
<td>IS, PCS</td>
<td>Enhanced expression cytokine and inflammatory genes</td>
</tr>
<tr>
<td>Sun, 2012(^{26})</td>
<td>IS, PCS</td>
<td>Activation RAAS and epithelial-to-mesenchymal transition, fibrosis, nephrosclerosis</td>
</tr>
<tr>
<td>Shimizu, 2012(^{37})</td>
<td>IS</td>
<td>Increase Klotho gene methylation, renal fibrosis</td>
</tr>
<tr>
<td>Sun, 2012(^{29})</td>
<td>IS, PCS</td>
<td>Increase renal tubular damage</td>
</tr>
<tr>
<td>Watanabe, 2012(^{45})</td>
<td>PCS</td>
<td>Induction mediators for fibrosis (TGF-(\beta) and Smad3)</td>
</tr>
<tr>
<td>Shimizu, 2013(^{43})</td>
<td>IS</td>
<td>Induction generation adhesion molecule (ICAM-1)</td>
</tr>
<tr>
<td>Bolati, 2013(^{30})</td>
<td>IS</td>
<td>Increased oxidative stress</td>
</tr>
<tr>
<td>(8) Whole kidneys</td>
<td>IS</td>
<td>Increase fibrosis</td>
</tr>
<tr>
<td>Adijang, 2008(^{40})</td>
<td>IS</td>
<td>Increase angiotsinogen expression</td>
</tr>
<tr>
<td>Shimizu, 2013(^{42})</td>
<td>IS</td>
<td>Increase angiotsinogen expression</td>
</tr>
<tr>
<td>(9) Hepatocytes</td>
<td>IS</td>
<td>Increase albumin production</td>
</tr>
<tr>
<td>Odamaki, 2004(^{52})</td>
<td>IS</td>
<td>Inhibition metabolic clearance losartan</td>
</tr>
<tr>
<td>Tsujimoto, 2010(^{32})</td>
<td>IS</td>
<td>Inhibition pumps involved in clearance of pravastatin</td>
</tr>
<tr>
<td>(10) Intestinal cells</td>
<td>IS</td>
<td>Increase albumin production</td>
</tr>
<tr>
<td>Tsujimoto, 2012(^{48})</td>
<td>IS</td>
<td>Inhibition pumps involved in clearance of pravastatin</td>
</tr>
<tr>
<td>(11) Adipocytes</td>
<td>IS</td>
<td>Increase insulin resistance, decrease lipogenesis, increase lipolysis, ectopic lipid redistribution</td>
</tr>
<tr>
<td>Koppe, 2013(^{34})</td>
<td>PCS</td>
<td>Increase insulin resistance, decrease lipogenesis, increase lipolysis, ectopic lipid redistribution</td>
</tr>
</tbody>
</table>

IS, indoxyl sulfate; ROS, reactive oxygen species; NO, nitric oxide; PCS, p-cresyl sulfate; OATs, organic anion transporters; RAAS, renin angiotensin aldosterone system; MCP-1, monocyte endothelial chemotactic protein-1; TGF-\(\beta\), transforming growth factor-\(\beta\); ICAM-1, intercellular adhesion molecule-1.
dose, and the type of model with regard to kidney function, fluctuating concentrations and different peak values might occur in the serum, which might, in turn, impact the interpretation of the observed biologic effect.

Third, our review data pointed overwhelmingly to a toxic effect of indoxyl sulfate and p-cresyl sulfate that may be skewed by submission bias. Investigators may be tempted not to finalize or publish studies showing no ill effect. Nevertheless, we retrieved five such studies by our analysis.52–56 Fourth, we should consider the possibility that we missed some studies in our analysis because of the approach followed, but more data would only increase the evidence delivered here.

However, our approach, based on rigid threshold levels, must also have excluded a number of interesting studies based on concentrations just above threshold77 and/or pathophysiologic key aspects highly relevant to the uremic syndrome, such as endothelial microparticle release,56 tissue factor activation,79 or metabolic capacity for glucuronidation.80

Because studies on toxicity in tubular cells suggest a relation to progression of kidney failure that is especially important in earlier stages of CKD, it can be argued that lower concentrations should be pursued in these experiments. However, even in dialysis patients, restraining progression may have a substantial clinical impact, because residual renal function is definitely inversely related to mortality.81 In addition, in most of studies on tubular toxicity that were retrieved,34,36–39,42,43,45,47,50 concentrations were in the same range as those concentrations found in patients with CKD stages 3–5 not on dialysis13,82 (i.e., lower than in dialysis patients).

In conclusion, a systematic retrieval approach searching for experimental studies evaluating the biologic (toxic) impact of the uremic solutes indoxyl sulfate and p-cresyl sulfate and taking into account precise criteria for concentrations that considered protein binding yielded 27 publications that conformed with the inclusion criteria.

Eleven of these studies obtained a high-quality score, indicating a substantial scientific impact. Most frequently, missing quality factors were insufficient number of experiments, lack of a dose–response analysis, and especially, absence of data showing the neutralization of the effect found by counteragents of the presumed mechanism. Future studies should comply with these conditions to be considered of high quality. The retrieved studies covered a broad array of effects, most of them related to clinically highly relevant aspects, such as cardiovascular disease or progression of CKD.

Table 4. Most important affected pathophysiologic mechanisms

<table>
<thead>
<tr>
<th>Affected Mediators</th>
<th>Effect</th>
<th>Toxin</th>
<th>Confirmed in Two or More Studies</th>
<th>Related to Inflammation</th>
<th>Related to Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Smooth muscle actin36,38,40,49</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Cytokine generation34,35</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>E-cadherin36,38</td>
<td>↓</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Nuclear Factor (erythroid-derived 2)-like 2-like 249,50</td>
<td>↓</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Extracellular-regulated kinase 1/244</td>
<td>↑</td>
<td>PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Fibronectin36</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Heme oxygenase-149,50</td>
<td>↓</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Intercellular Adhesion Molecule-147</td>
<td>↑</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Inflammatory genes34</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Insulin receptor substrate-144</td>
<td>↓</td>
<td>PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Klotho39</td>
<td>↓</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Mitogen-activated protein kinase: MEK 1/2; p3835</td>
<td>↑</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Multidrug resistance-associated protein-248</td>
<td>↓</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>NADPH oxidase27,30,45</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Nuclear Factor-κB35</td>
<td>↑</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Organic Anion Transporters40,48</td>
<td>↓</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Osteoblast-specific proteins40</td>
<td>↑</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Phosphoinositide 3-kinase44</td>
<td>↓</td>
<td>PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Protein kinase B/Akt44</td>
<td>↓</td>
<td>PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Renin-angiotensin-aldosterone system36</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>E-selectin30</td>
<td>↑</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Senescence proteins41a</td>
<td>↑</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Smad2/3 and Smad436,43</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Snail36</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Tissue factor46</td>
<td>↑</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Transforming Growth Factor-β36,40,43,49</td>
<td>↑</td>
<td>IS, PCS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Zonula occludens-138</td>
<td>↓</td>
<td>IS</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

IS, indoxyl sulfate; PCS, p-cresyl sulfate.

aSenescence proteins: senescence-associated β-galactosidase, 16INK4a, p21WAF1/CIP1, p53, and retinoblastoma protein.
solutes. This knowledge may be supportive of the development of strategies to lower the concentration of these solutes in the body.

CONCISE METHODS

The study was limited to indoxyl sulfate and p-cresyl sulfate, the two protein-bound uremic toxins that have been most extensively evaluated until now. A literature search using Pubmed through Reference Manager was undertaken with the following terms: p-cresyl sulfate or p-cresylsulfate, or indoxylsulfate or indoxyl sulfate. This search was completed with additional references emanating from the originally retrieved publications. The search contained all obtained references with June 30, 2013, as closing date (Figure 1).

We only included primary studies assessing the biologic effect of indoxyl sulfate and/or p-cresyl sulfate in vitro or in animals where an acceptable free concentration was present. For this reason, analytical studies, clinical concentration or outcome studies, and studies on the origin and generation of the compounds of interest, medical interventions to decrease their concentration, and removal by dialysis or other strategies were all excluded. Also, studies not specifying the pursued solute concentrations or unclear on the applied methods, making reproduction of the experiments...
difficult, were rejected as well as reviews, abstracts, and studies referring to conditions other than CKD, including AKI.

Only studies conforming to the preset target concentrations, defined as values not exceeding the highest levels obtained in uremia (reported in Table 1), were included. Additional conditions for accepting a study were as follows: in vitro studies with use of medium containing concentrations of albumin corresponding to real-life in vivo conditions or animal studies with injection or oral administration of the molecules of interest or their precursors and the appropriate concentration of toxin and serum albumin. In the absence of albumin in vitro, the use of solute concentrations had to be in accordance with reported free toxin concentrations, which is also detailed in Table 2.

Based on this approach, two investigators independently selected the studies and extracted all data, with discrepancies resolved by a third investigator (Figure 1). The studies were then checked for their content using a data extraction list conceived as reported in Supplemental Material 1, with specific attention to the experimental setting, the assessed problem, the molecule of interest, the applied concentrations, the presence (or absence) of appropriate concentrations of albumin, the involved pathways, and the successful application of neutralizing interventions.

From our analysis, it became clear that the quality of the retrieved publications was not always the same. However, to the best of our knowledge, there are no checklists available for evaluating quality or reliability of primary studies of biologic effects, the primary target of our search. Thus, we developed a quality score (Supplemental Material 2) that was given to each study based on the number of experiments (1 point if six or more experiments); confirmation of the obtained results by an alternative approach (same parameter, different methods, same pathway, different parameters; 1 point); proof of neutralization of the basic identified mechanism by the appropriate antibodies, counteractive agents, or other interventions (1 point); use of a real-life model (1 point; here, we excluded studies in specific genetic animal strains instead of wild type, especially because results between these two groups in our search were not always confirmed20,40,42,49; also, specific cell types that are not necessarily representative of real-life conditions were excluded [e.g., human umbilical vein endothelial cells], because the phenotype of venous endothelium

Figure 2. Continued.
does not always match that of the arterial cell, which is where uremic vascular damage and atherosclerosis essentially take place\(^5\); and finally; performance of dose–response experiments but only if the other conditions were appropriate (in the presence of albumin or in the free concentration range; 1 point). Thus, a maximum quality score of 5 points could be obtained.

These quality scores were attributed independently by two of the authors and matched and double-checked by a third author in case of disagreement.

Because there are substantial ethical and practical obstacles to performing dose–response experiments for \textit{in vivo} animal studies, 1 extra point was added to all publications containing animal studies as long as the same publication did not include \textit{in vitro} dose–response studies as well.

**ACKNOWLEDGMENTS**

We thank Mrs. Caroline Vinck for linguistic correction of this publication.

**DISCLOSURES**

None.

**REFERENCES**


Uremic Toxicity of IS and pCS
BRIEF REVIEW

www.jasn.org


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013101062/-/DCSupplemental.
**SUPPLEMENTAL DATA 1**

**Adijang et al Nephrol Dial Transplant - 2008**

Model: study of aortic calcification (morphologic and immunohistochemic characterization of osteoblast related proteins (osteopontin, core binding factor 1 (Cbfal), alkaline phosphatase (ALP), osteocalcin), indoxylsulfate (IS) and organic anion transporter 1 and 3 (OAT 1 and 3); morphology of kidneys (glomerular area, mesangial area, MT-positive tubulointerstitial area, TGF-βI-positive glomerular area and TGF-βI-positive tubulointerstitial area).

Setting: in vivo in Dahl salt resistant rats and in salt sensitive Dahl rats, treated or not by administration PO of indoxylsulfate for 30 weeks (no IS treated salt resistant group)

Toxin: indoxylsulfate; PO administration to rats (200 mg/kg)

Concentration: 23.1 +/- 3.6 mg/L at the end of the administration period (30 weeks – lower before).

Albumin concentration: Not measured, as can be expected in rats.

Pathophysiologic changes:

- Increase in aortic thickness and calcification. Increase in osteoblast markers, IS and OATs.
- Increase of glomerular area, mesangial area, MT-positive tubulointerstitial area, TGF-βI-positive glomerular area and TGF-βI-positive tubulointerstitial in IS treated Dahl rats (n=10 – biochemical results in only 6)

Note: No salt resistant rats with IS administration

**Adijang et al Biochem Biophys Res Commun - 2010**

Model: Study of cell senescence evaluated by immunohistochemistry of senescence-associated β-galactosidase (SA-β-gal), and senescence-related proteins such as p16INK4a, p21WAF1/CIP1, p53 and retinoblastoma protein (Rb).

Setting: in vivo in Dahl salt resistant rats and in salt sensitive Dahl rats, treated or not by administration PO of indoxylsulfate-for 32 weeks.

Toxin: indoxylsulfate; PO administration to rats (200 mg/kg)

Concentration: In indoxylsulfate treated Salt sensitive Dahl rats, indoxylsulfate is continuously fluctuating between 15 and 20 mg/L between weeks 8 and 32, with the highest values around 20 exactly at these two moments. Values are somewhat lower in non-sensitive rats, between 14 and 9.

Albumin concentration: Not measured, as can be expected in rats.

Pathophysiologic changes:

- Increase in β-galactosidase (SA-β-gal), 16INK4a, p21WAF1/CIP1, p53 and retinoblastoma protein (Rb) in IS-treated Dahl sensitive rats as compared to Dahl-sensitive rats who were not treated by IS (n=8).—Also aortic calcification and wall thickness more important than in non-IS treated rats.
Note: Dahl sensitive rats not receiving IS showed the same lesions but significantly less pronounced than the IS treated Dahl sensitive rats. Salt-resistant rats receiving indoxylsulfate showed no significant changes.

Bolati et al Am J Nephrol - 2011

Model: Study of epithelial to mesanchymal transition in rat kidneys and cultured proximal tubular cells by immunohistochemistry, reverse transcription PCR and immunoblotting for epithelial markers E-cadherin and zonula occludens-1 and mesenchymal marker α-SMA. In vivo tests on normal and Dahl sensitive hypertensive rats.

Setting: in vitro and in vivo

Toxin: indoxylsulfate

Concentration: in vivo 9.4 +/- 1.3 mg/L (normal rats) and 18.9 +/- 2.6 (Dahl rats). In vitro 250 µM (thus too high).

Albumin concentration: in vivo albumin as in living rats; in vitro 10% FBS

Pathophysiologic changes:

Reduction of expression of E-cadherin and ZO-1 in both normal and Dahl rats treated by indoxylsulfate and enhanced expression of α-SMA in vivo (n= 6). Similar results in vitro (n= 3 to 5).

Note: -

Bolati et al BMC Nephrol 2013

Model: In both wild type and salt sensitive hypertensive Dahl rats, IS-treated vs non-IS treated equivalent; study on proximal tubular cells; study of anti-oxidant regulators: erythroid-derived 2 like 2 (Nrf2); heme oxygenase-1 (HO-1; NAD(P)H quinine oxide reductase (NQO1); and of 8-hydroxy-deoxyguanosine (8OHdG), marker of ROS activation.

Setting: Dahl wild type, IS vs. no IS

Toxin: indoxylsulfate

Concentration: 9.4 mg/dL.

Albumin concentration: as in rats.

Pathophysiologic changes:

Only in IS treated wild type:
- Suppression of anti-oxidant Nrf2 and HO-1 and activation of 8OHdG vs. non-IS treated.
- According to the data for IS-treated Dahl sensitive rats no significant difference from non treated. Thus only wild type data relevant.
Note: Study contains in vitro data but not appropriate. Also animal study in a second model, Sprague-Dawley with CKD (thus not IS treated) with neutralization by AST-120

**Chitalia et al Circulation - 2013**

Model: assessment of de-endothelialized vascular smooth muscle cells for tissue factor expression, activity, stability and posttranslational modification. Changes were associated to clot formation.

Setting: Uremic serum and IS induce tissue factor generation; this effect is related to clot formation. TF breakdown through ubiquination and this process is slowed down by indoxylsulfate. Experiments occurred in a flow loop mimicking coronary circulation.

Toxin: indoxylsulfate

Concentration: IS 25 mg/L.

Albumin concentration: Presumably 4 g/L, email confirmation by author.

Pathophysiologic changes:

Activation of tissue factor generation (n=3). Effect seen from 4 hours on, continued over 24 hours. Also increasing effect with increasing dose of IS. SMC injury further enhances the effect.

Note: Same effect as with uremic toxins also seen with uremic serum; effect could be reduced by anti-TF neutralizing antibody. Also similar effect for uric acid and indole acetic acid.

**Dou et al KI - 2004**

Model: endothelium (HUVEC)

Setting: in vitro

Toxin: indoxylsulfate

Concentration: 25-250 mg/L

Albumin concentration: 4%

Pathophysiologic changes:

- Inhibited endothelial proliferation (5-bromo-2-deoxyuridine incorporation) (BrdU) (25-250 mg/L) (dose response) (n=4)

- Inhibited endothelial wound repair after injury (videomicroscopy) (125-250 mg/L – if with albumin) (dose response) (n=?)

Note: similar results reported with p-cresol, but p-cresol is not present as such in the body [Martinez, 2005 339 /id;de, 2005 322 /id;Vanholder, 2011 321 /id]; no induction of endothelial apoptosis (annexin-V testing by flow cytometry). Effect less pronounced here in presence than in absence of albumin.
**Dou et al J Thromb Haemost - 2007**

Model: endothelium (HUVEC)

Setting: in vitro

Toxin: indoxylsulfate

Concentration: 125-250 mg/L

Albumin concentration: 4% (g/dL)

Pathophysiologic changes:

- Induction of reactive oxygen species (ROS) measured by cytofluorometry (125-250 mg/L) (dose response) (n=10)
- Inhibition of NAD(P)H-oxidase neutralized this pro-inflammatory effect whereas inhibitors of xanthine oxidase, NO-synthase and mitochondrial RO production had no effect
- Indoxylsulfate strongly inhibited glutathione, one of the most important antioxidant systems in the cell. This test was not performed in the presence of albumin, though

Note: inhibition of the pro-oxidant effects by the antioxidants Vit C, vit E and N-acetylcysteine (NAC)

**Faure et al J Thromb Haemost - 2006**

Model: endothelium (HUVEC)

Setting: in vitro

Toxin: indoxylsulfate

Concentration: 256 mg/L

Albumin concentration: 4% (g/dL)

Pathophysiologic changes:

- Increase in number of released endothelial microparticles as detected by enhanced annexin-V labeling measured by flow cytometry (n=8)

Note: -

**Ito et al J Biol Chem - 2010**

Model: leukocyte endothelial interaction

Setting: in vivo studies after indoxylsulfate administration in drinking water in 5/6 nephrectomized mice; controls: 5/6 nephrectomized without indoxylsulfate

Toxin: indoxylsulfate
Concentration: 15.7 mg/L

Albumin concentration: no serum albumin mentioned but should be the usual ones for in vivo conditions

Pathophysiologic changes:

Interaction of leukocytes with endothelium of femoral arteries is enhanced in intravital microscopy studies (n=5)

Quantitative real-time PCR analysis also showed an increase of mRNA expression of E-selectin and the NAD(P)H oxidase subunits p47phox and p22phox vs normal rats and for E-selectin also vs nephrectomized rats not treated with indoxylsulfate (n=5). No (significant) changes however for ICAM-1 and VCAM-1

Note: the study contains an in vitro setting where no appropriate albumin is added and an in vivo setting in mice, where by definition appropriate albumin should be present. Injection of anti-selectin-E antibody neutralizes the interaction. Concentration IS low as compared to human uremia.

**Itoh et al Anal Bioanal Chem - 2012**

Model: endothelium (HUVEC)

Setting: in vitro

Toxin: indoxylsulfate

Concentration: 29.9 and 57.2 mg/L

Albumin concentration: 4% (g/dL)

Pathophysiologic changes:

Increase in endothelial ROS production compared to control (no toxin) (n=3)

Note: p-cresylsulfate no effect in absence of albumin; concentration 37.1 and 109.1 mg/L

**Koppe et al J Am Soc Nephrol - 2013**

Model: evaluation of several aspects of insulin resistance in vivo-in mice and in vitro on cultured adipocytes (modified fibroblasts) and C2C12 myotubes (equivalent to myocytes after modification of C2C12 myoblasts).

Setting: in vivo assessment in PCS treated vs control mice of glucose response to insulin, insulin-induced phosphorylation of PKB/Akt and activation of phosphorylation of ERK1/2 in gastrocnemius, adipose tissue size, ectopic lipid distribution; in vitro assessment in presence of PCS compared to control in adipocytes of lipogenesis and lipolysis, in myotubes of glucose uptake in response to insulin as well as elements of the insulin pathway by Western blotting and ERK1/2 phosphorylation.

Toxin: p-cresylsulfate.
Concentration: In vivo 5.01 +/- 1.59 mg/dl total and 0.82 +/- 0.24 free (total OK with clinical reality, free 2.5 times above mean uremic concentration). In vitro 40 mg/l (OK).

Albumin concentration: In vivo not measured, as can be expected in healthy mice (but protein binding in the expected range: 90.5 +/- 2.0 %). In vitro albumin 35 g/l BSA.

Pathophysiologic changes:

In vivo with PCS: decreased glucose response to insulin (n= 5-8), inhibited insulin-induced phosphorylation of PKB/Akt and activation of phosphorylation of ERK1/2 in gastrocnemius (n= 4-6), decreased adipose tissue size (n = 8), increased ectopic lipid distribution (n=8); in vitro in presence of PCS: in adipocytes decreased lipogenesis and increased lipolysis (n=4), in myotubes inhibition of glucose uptake in response to insulin together with changes in the following elements of the insulin pathway (Western blotting) - serine 473 phosphorylation of PKB/Akt, tyrosine-phosphorylation of IRS-1, serine phosphorylation (Ser 636) of IRS-1, p85 subunit of PI3K coimmunoprecipitation: (n=4-5), and ERK1/2 phosphorilation (n=6).

Note: Probenecid prevents PCS-induced disruption of insulin signaling pathways in C2C12 myotubes; the prebiotic AXOS decreased p-cresylsulfate levels and corrected metabolic changes.

**Lekawanvijit et al Eur Heart J - 2010**

Model: Cardiac fibroblast collagen synthesis, cardiomyocyte proliferation (^3H-proline and ^3H leucine incorporation). In THP1 cells (substitute for monocytes – leukemia celline) production of cytokine TNF- α, IL-6, IL-1β mRNA expression estimated by PT-PCR. Furthermore, estimation of activity of MAPKinase and NFκB.

Setting: in vitro

Toxin: indoxylsulfate

Concentration: fibroblast collagen synthesis from 3µM on; on myocyte hypertrophy from 0.01 µM on (i.e. below the normal free concentration); TNF-α and IL-1β 100 µM (too high); IL-6 1 µM; MAPKinase and NFκB: 10 µM.

Albumin concentration: myocytes and fibroblasts in 10% FB); TMP-1 in 0.5% BSA.

Pathophysiologic changes:

IS had pro-fibrotic (n=9 in triplicate), pro-hypertrophic (n=5 in triplicate)and pro-inflammatory effects (n=3). Decreasing IS might help in decreasing cardio hypertrophy in CKD. In THP1 cells increased production of TNF- α, IL-6, IL-1β mRNA expression estimated by PT-PCR. Furthermore activation of MAPKinase and NFκB (n= 3).

Note: neutralization by inhibition of RWJ-67657 and U0126, linked to p38 and MEK1/2 pathways
**Odamaki et al Nephrol Dial Transplant - 2004**

Model: In vitro study of albumin production by isolated hepatocytes.

Setting: this is a study of several different compounds. The indoxylsulfate experiments were performed in the presence and absence of albumin.

Toxin: indoxylsulfate

Concentration: 50 and 100 mg/L.

Albumin concentration: 4 g/dL.

Pathophysiologic changes:

Increase in albumin production.

Note: is in fact not a toxic but a positive effect. No difference with and without albumin.

**Schepers et al Nephrol Dial Transplant - 2007**

Model: leukocyte function (whole blood)

Setting: in vitro (healthy volunteers)

Toxin: P-cresylsulfate

Concentration: 121.0 mg/L

Albumin concentration: no serum albumin mentioned but should be the usual ones for in vivo conditions.

Pathophysiologic changes:

Increase of oxidative burst activity of baseline leukocytes (monocytes and lymphocytes) (n=10); granulocytes trend but not significant.

Note: Concentration P-CS slightly higher than accepted maximum.

**Shimizu et al Life Sci - 2012**

Model: Study in proximal tubular cells on the effect on the expression of Monocyte Chemotactic Protein-1 (MCP-1), a pro-inflammatory agent responsible for recruiting leukocytes into tubulointerstitial tissue. This study was performed in salt sensitive hypertensive Dahl and wild type rats to whom indoxylsulfate was administered; the rats were not made renal insufficient. Furthermore also in vitro study in tubular cells of the effect on activation of ERK, p38, JNK, NF-κB and p53. In addition, study of the effect in absence and presence of an antioxidant and inhibitors of the above mediators.

Setting: in vitro and in vivo

Toxin: indoxylsulfate
Concentration: in vivo, in normal rats given indoxylsulfate 9.4 +/- 1.4 mg/L, in Dahl rats 18.9 +/- 2.8 (appropriate thus). In vitro 250 µMol (thus too high).

Albumin concentration: in vivo, serum albumin as can be expected in rats, in vitro 10% FBS

Pathophysiologic changes:

Induction of expression of MCP-1 at relevant concentrations in indoxylsulfate treated rats, both normal (n= 5) and Dahl (n= 5). All other experiments at inappropriate concentrations.

Note: indoxylsulfate administered per os (200 mg/kg in drinking water).

Shimizu et al Am J Physiol Cell Physiol - 2013

Model: In vitro and in vivo study on renal tubular cells: expression of angiotensinogen and cAMP response element-binding protein (CREB).

Setting: in vivo in Dahl salt sensitive rats for expression of angiotensinogen.

Toxin: indoxylsulfate; PO administration to rats (indoxylsulfate concentration: 9.4 mg/L +/- 1.3.

Concentration: In vivo: indoxylsulfate concentration: 9.4 mg/L +/- 1.3. In vitro experiments (all the rest) were at too high concentrations (250 µM) for protein-poor medium (10% FBS).

Albumin concentration: Not measured, as can be expected in rats.

Pathophysiologic changes:

Increase in angiotensinogen expression (n=8).

Note: -

Shimizu et Am J Nephrol - 2013

Model: evaluation in proximal tubular cells of the p53-TGF-β1-Smad3 pathway, involved in inducing fibrosis.

Setting: In vivo study on proximal tubular cells of salt resistant and salt sensitive Dahl hypertensive rats. Also in vitro studies but with only 10% FBS and for that protein concentration too high indoxylsulfate (250 µM).

Toxin: indoxylsulfate; PO administration to rats (indoxylsulfate concentration: 9.4 mg/L +/- 1.3.

Concentration: In vivo: indoxylsulfate concentration: 9.4 mg/L +/- 1.3. In vitro experiments (all the rest) were at too high concentrations (250 µM) for protein-poor medium (10% FBS).

Albumin concentration: Not measured, as in rats.

Pathophysiologic changes:

Only morphologic changes (increase TGF-β1 and Smad3 positive area). No statistical data. (n=8).
Note: -

Shimizu et al Life Sci - 2013

Model: assessment of renal regulation of ICAM-1, playing a role in attracting monocytes and macrophages.

Setting: Next to human tubular cells (HK-2) studied in vitro, also in vivo studies were performed with Dahl rats receiving IS. Two strains were receiving IS: Dahl salt sensitive rats and wild type. Rats received IS (200 mg/kg) for 32 weeks.

Toxin: indoxylsulfate

Concentration: in vivo assessment: 9.4 mg/L in wild strain and 18.9 mg/L in Dahl hypertensive rats; in vitro: 250 µmol but without albumin, only Dulbecco. Thus only in vivo experiments relevant.

Albumin concentration: As can be expected in normal rats.

Pathophysiologic changes:

Assessment of ICAM-1 mRNA expression in both wild strain and salt sensitive rats treated with IS (n=5) (also Dahl hypertensive rats not receiving IS showed higher ICAM-1). Dahl hypertensive treated with IS had higher expression than Dahl hypertensive and no IS.

Note: Inhibitors of NADPH oxidase, NF-κB and p53 suppressed the increase of ICAM-1 mRNA expression (but only in vitro).

Sun et al Nephrol Dial Transplant - 2012

Model: Inflammatory gene expression in cultured renal proximal tubular cells

Setting: in vitro

Toxin: indoxylsulfate and p-cresylsulfate

Concentration: IS and PCS 1 and 5 mg/L (concentrations thus correspond to free fraction)

Albumin concentration: Serum starved cells

Pathophysiologic changes:

Stimulation of a whole series of pro-inflammatory genes (see below) (n=?)

Note: The major genes from cytokines in the functional networks that were activated were Tgfβ1, fasl, IL6/15, IL15, Csf1/3 and Cxcl10; those from the triggered intracellular signals were Stats, Smads, Nfkb2, Ikbkb, Bcl2 and Bax. Functional networks linked to Tgfβ1: Col4a5, Cxc10, Fasl, Stat1 and Ikbkb.
Sun et al PLOS One - 2012

Model: Study of activation of the renal renin–angiotensin–aldosterone system (RAAS) in proximal tubular cells and of renal tubular epithelial-to-mesenchymal transition (EMT) and of TGF-β, Snail, fibronectin, α-Smooth muscle actin (α-SMA) and E-cadherin production as mechanisms in the induction of kidney fibrosis. Direct study of fibrosis by assessment of nephrosclerosis scores.

Setting: in vitro and in vivo

Toxin: indoxylsulfate and p-cresylsulfate

Concentration: in vitro studies were showing a positive effect from 1 mg/L on for both IS and PCS with a further dose responsive increase for 5 and 50 mg/L. The in vivo data (TGFβ, Snail, fibronectin α-SMA and E-cadherin) were collected after peritoneal injection in mice but plasmatic level was not specified.

Albumin concentration: in vitro PBS, % not specified; in vivo as in animals.

Pathophysiologic changes:

Activation of RAAS , EMT , TGF-β , Snail , TGF-β , fibronectin , and α-SMA and decrease of E-cadherin production . Increase of nephrosclerosis , all with both IS and PCS (n=8).

Note: Neutralization effect with losartan.

Sun et al Kidney Int - 2012

Model: study of kidney fibrosis, methylation of Klotho gene, Klotho expression.

Setting: in vivo in B-6 mice; in vitro on renal tubular cells

Toxin: indoxylsulfate and p-cresylsulfate; in vivo injected to mice

Concentration: for indoxylsulfate in mice treated by indoxylsulfate 8.55±0.37,mg/L, treated by p-cresylsulfate 5.61±0.60 mg/l (corresponding to a very moderate increase in concentration); for p-cresylsulfate in mice treated by pCS 1.82 +/-0.33 and in those treated by IS 0.66 +/-0.04 which are in fact numbers corresponding to normal. In vitro 1, 5 and 50 mg/L ( 1 (pCS) and 5 (IS) OK for free fraction).

Albumin concentration: in vivo albumin as in living mice; in vitro no protein content specified.

Pathophysiologic changes:

In mice (n= 8) induction of kidney fibrosis, hypermethylation of the Klotho gene and decreased Klotho expression; inhibition of DNA methyltransferase isoform 1 caused a decrease of methylation and increased Klotho expression in vitro.

Note: In mice treated by one toxin the other toxin rises as well, in absence of renal failure.
Tsujimoto et al J Pharm Pharmacol - 2010

Model: metabolic clearance of losartan – measurement of the metabolite EXP-3174 using pooled human liver microsomes

Setting: in vitro

Toxin: indoxylsulfate

Concentration: Indoxylsulfate is diluted in uremic serum at 3-300 µmol/L and then diluted into the medium with microsomes 10% vv. The concentration in normal serum is within the acceptable range. As the sample is diluted 1/10 the protein binding will certainly decrease, but the final concentration of 20 µmol remains well within the range also for free solute. An effect is seen at 300 µmol/L, thus 30 µmol/L diluted (as a matter of fact a bit too high for entirely free, but there is still some protein).

Albumin concentration: Final albumin concentration low but total concentration within the acceptable range of free concentration.

Pathophysiologic changes:

Inhibition of losartan metabolism (dose response but only starting at the uremic range, n=3 or 4) (uremic range is the highest concentration of the dose-response)

Note: Studies also on other toxins; for the cresols, however, only p-cresol was studied (of course, very likely, some cresol reaches the liver)

Tsujimoto et al Ther Apher Dial - 2012

Model: Investigation of pump systems involved in the non-renal clearance of pravastatin, in view of its accumulation in renal failure, in spite of it not being cleared by the kidneys: assessment of the role of uremic toxins and uremic milieu on the expression of OATP2B1 (at play in intestinal uptake of Pravastatin) and MRP-2 (at play in intestinal/bile secretion of Pravastatin) in Caco-2 cells (intestinal cell line) and of OATP1B1 and OATP2B1 (at play in intestinal and hepatic uptake of pravastatin) in HEP3B cells (hepatic cell line).

Setting: This is a pure in vitro study using HBSS (Hank’s balanced salt solution) as medium. The latter contains no protein or albumin but experiments were done at low enough concentrations to be representative for free fraction.

Toxin: indoxylsulfate

Concentration: 20 µmol, which corresponds to free concentration.

Albumin concentration: 10% FCS.

Pathophysiologic changes:

Clear inhibition only in Caco-2 for OATP2B1 and MRP-2 (n=3). No consistent changes in HEP3B (only slight decrease for OATP2B1.
Note: inhibitory effect not only seen for IS but also for IAA, hippuric acid and cmpf. No significance of effect given. Two effects taken together seem contradictory.

**Watanabe et al Kidney Int - 2013**

Model: evaluation of toxicity on renal tubular cells via activation of production of radical oxygen species (ROS).

Setting: in vitro assessment of activity of NADPH-oxidase; upregulation mRNA inflammatory mediators and TGF-β1 (associated with renal fibrosis) – cell cultures in K-SFM (keratinocyte serum free medium; no albumin); in vivo administration p-CS to 5/6 nephrectomized rats for 4 weeks with assessment of tubular damage and enhancement oxidative stress.

Toxin: p-cresylsulfate.

Concentration: in vitro: effect seen only at 100 and 500 µmol/L (thus too high in absence of protein in medium); no significant effect at 10 µmol/L (which is a correct concentration without protein). In vivo (5/6 nephrectomized rats): animals evolved with or without CKD till week 16, then one randomly selected CKD group received PCS up till 20 weeks, the other not. At week 16 (in all rats) PCS was between 2.0 and 2.99 µmol/L; at week 20 (non-PCS treated rats) PCS was 4.52 +/- 5.29 µmol/L; in the PCS treated rats this value was 32.57 +/- 19.94 µmol/L. 32 µmol/L is in the low human uremic range; 5 µmol/L is in the low normal range.

Albumin concentration: Not measured, as in rats.

Pathophysiologic changes:

Only in vivo changes count: in the presence of added PCS, an increase of tubule degeneration, TGFβ1 activity, free radical production, and NADPH-oxidase activity were observed. (N=9)

Note: Effect neutralized by Knock-down of p22^phox^ and p22 neutralized the effect pointing to the importance of NADPH-oxidase. Also suppression effect by administration of probenecid and NAC. Mechanism similar as for indoxylsulfate. After 4 weeks of PCS not only PCS levels but also Scree and urea were slightly but significantly higher (pointing to a possible broader general uremic effect in this group, but then still PCS-induced – although the differences on PCS are much more dramatic than those in urea or creatinine; changes just point to an impact on kidney function.

**Yisireyili et al Life Sci - 2013**

Model: In Dahl salt sensitive hypertensive rats, investigation of heart weight, left ventricular weight, diameter of cardiomyocytes, parameters of cardiac fibrosis [Mason-trichrome, TGFβ1, α-SMA, type 1 collagen] and of oxidative stress [NADPH-oxidase (Nox4), malondialdehyde (MDA, 8-hydroxydeoxyguanosine (8OHdG]]; finally also expression of anti-oxidant regulators, constituting nuclear factor [(erythroid-derived 2) like 2 (Nrf2); heme oxygenase-1 (HO-1).

Setting: In vivo study on Dahl hypertensive rats administered IS compared to same rats not administered IS
Toxin: indoxylsulfate
Concentration: 18.9 mg/L.
Albumin concentration: as in rats.

Pathophysiologic changes:

In IS-treated rats:
- Increase in diameter cardiomyocytes
- Increased fibrotic area; increased staining for TGFβ1, SMA, type-1 collagen,
- Increase of Nox4, 8-OHdG and MDA
- Decreased staining for Nrf2 and HO-1

Note: no effect IS in wild type rat; study contains no in vitro arm

Yamamoto et al Kidney Int - 2006
Model: In vitro study of the effect of indoxylsulfate on smooth muscle cell proliferation.

Setting: although most experiments are in the absence of albumin, some have been performed in the presence of albumin

Toxin: indoxylsulfate
Concentration: 250 and 500 µM.
Albumin concentration: 4 g/dL.

Pathophysiologic changes:
Increased smooth muscle cell proliferation.

Note: data with and without albumin are expressed differently (fold-change vs. absolute values) making comparison difficult. Fold changes seem at least as prominent in the presence as in the absence of albumin.

Model: Proliferation, senescence and production of nitric oxide and oxygen free radicals by HUVEC as a model of endothelial damage; cell proliferation: incorporation of ³H-thymidine and by direct cell counting; cell senescence: in situ staining for SA-β-galactosidase

Setting: in vitro

Toxin: indoxylsulfate
Concentration: 1.25 to 125 mg/L; effect from 2.5 mg/L on
Albumin concentration: No mention of albumin

Pathophysiologic changes:
Inhibition of proliferation; enhancement of senescence; decrease NO production; increase ROS production (for 2.5 mg/L from the 3d hour on) (n= 4 in duplicate)

Note: This in vitro study is associated with an observational clinical study showing an in vivo improvement of endothelial function as demonstrated by flow-mediated endothelium-dependent vasodilatation (FMD) after 24 weeks of AST-120. This study arm was observational. There were no controls. All 40 patients received AST-120. Correction of in vitro effects by probenecid and the antioxidants NAC, rotenone and apocynin.
**SUPPLEMENTAL DATA 2**

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