Original Articles

$p$-Cresyl sulphate has pro-inflammatory and cytotoxic actions on human proximal tubular epithelial cells

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ABSTRACT

Background. $p$-Cresyl sulphate ($p$-CS) and $p$-cresyl glucuronide ($p$-CG) are uraemic toxins that exhibit pro-inflammatory features in leukocytes and are associated with the progression of chronic kidney disease (CKD). Tubular cells are key targets of nephrotoxic agents and tubular cell death and activation contribute to the progression of CKD. However, the potential toxicity of these compounds on tubular cells is not fully understood. More specifically, apoptosis has never been studied.

Methods. HK-2 human proximal tubular epithelial cells were studied. Cell death was evaluated by flow cytometry of DNA content and by morphology. Gene expression was studied by real-time (RT)-PCR. Protein expression was studied by western blot and flow cytometry.

Results. Long-term (7 days) exposure to $p$-CS induced apoptosis in HK-2 cells in a concentration-dependent manner. In addition, short-term (3 h) exposure to $p$-CS promoted the expression of the TWEAK receptor Fn14, cooperated with TWEAK in promoting cell death and increased inflammatory gene expression. Albumin was cytotoxic and increased the inflammatory response to $p$-CS concentrations found in the circulation of non-dialysis CKD patients. In contrast, no biological actions of $p$-CG were observed on HK-2 cells, either alone or in combination with $p$-CS.

Conclusions. This study demonstrates for the first time that $p$-CS has pro-apoptotic and pro-inflammatory effects on tubular cells. These results identify mechanisms by which uraemic toxicity may contribute to CKD progression.

INTRODUCTION

Uraemic toxins are molecules excreted or metabolized by normal kidneys that progressively accumulate in chronic kidney disease (CKD) and exert an adverse biological effect on a biological system [1–3]. Uraemic toxicity derived from the accumulation of these molecules has received attention as both a potential cause for the excess of cardiovascular disease and mortality observed in CKD and as a potential contributor to the progression of CKD itself [4, 5]. Among the uraemic retention solutes, protein-bound compounds such as the $p$-cresol conjugates $p$-cresyl sulphate ($p$-CS) and $p$-cresyl glucuronide ($p$-CG) have attracted most interest in recent years due to their poor clearance by conventional dialysis and their potential toxicity [6].
Observational studies have linked systemic inflammation to cardiovascular events and mortality in CKD [7]. p-CS is pro-inflammatory by activating leukocyte free radical production [8, 9] and plays a role in endothelial microparticle release, an indirect indicator of vascular damage [10]. In contrast, p-CG per se exerts no pro-inflammatory activity on leukocytes, but it enhances free radical generation induced by p-CS in these cells [9], indicating the potential for synergism among uraemic toxins.

A hallmark of advanced CKD is progression, even when the original cause of CKD is no longer active. The molecular mechanisms have not been totally unravelled. Local factors such as loss of parenchymal renal cells, the presence of inflammatory cells and fibrosis, activation of kidney cells and infiltrating leukocytes, haemodynamic factors and others may have a role [11]. In this regard, there is evidence for a role of uraemic toxins in the process. The use of the intestinal adsorbent AST-120 that binds uraemic toxins and the use of peritoneal dialysis as renal replacement therapy have been associated with slower progression of CKD [12–16]. Peritoneal dialysis is associated with lower toxin concentration than haemodialysis [17–19]. Decreased tubular injury has been described in AST-120-treated patients, suggesting that tubular cells may be targets of uraemic toxins [16]. In this regard, p-CS levels predict CKD progression [5]. p-CS may also be involved in renal fibrosis via epithelial-to-mesenchymal transition induced by the renin–angiotensin system [20]. Further studies also revealed that p-CS suppresses the transcription of the renal-secreted hormone Klotho through hypermethylation of the gene in renal tubular cells [21].

We have now explored the effects of the uraemic toxins p-CS and p-CG on cultured human proximal tubular cell apoptosis and inflammatory phenotype. p-CS, but not p-CG, was found to enhance apoptosis. In addition, p-CS, but not p-CG, promoted a pro-inflammatory phenotype in human tubular cells.

METHODS

Cells and reagents

HK-2 human proximal tubular epithelial cells (ATCC, Rockville, MD) were grown on Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite and 5 ng/mL hydrocortisone in 5% carbon dioxide at 37°C [22]. For experiments, cells were rested in serum-free media 24 h prior to the addition of p-CS, p-CG or their respective control salts and throughout the experiment. Ten thousand cells were seeded for flow cytometry experiments, while 200,000 cells were seeded for RNA extraction and western blot. Bovine serum albumin (Sigma-Aldrich, Co., St. Louis, MO) was added at a concentration of 40 mg/mL in some experiments. This is the concentration found in serum and may be present in proximal tubular fluid in proteinuric states.

Two experimental settings were explored: (i) addition of toxins or vehicle and collection of cells or supernatants at 3 to 48 h, (ii) addition of fresh toxin- or vehicle-containing medium every 48 h and collection of cells at Day 7.

The p-CS potassium salt and the p-CG ammonium salt were synthesized and purified as previously reported [9]. The purity of both p-CS and p-CG was checked by liquid chromatography–mass spectrometry and nuclear magnetic resonance. The concentrations at which both compounds were added ranged from 1 to 500 μg/mL in the case of p-CS and 25 or 50 μg/mL for p-CG, with 105 and 24 μg/mL being the maximum serum concentrations found in a haemodialysis population [9]. Equimolar potassium chloride and ammonium chloride solution were used as control (vehicle).

Cell death

Apoptosis was characterized by morphological and functional criteria. Nuclei of formalin-fixed cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) to observe the typical morphological changes, as previously described [23]. For assessment of apoptosis by flow cytometry, 10,000 cells were seeded in 12-well plates (Costar, Cambridge, MA) in 10% fetal calf serum (FCS) RPMI overnight. They were rested in serum-free medium for 24 h. Thereafter, p-CS or p-CG was added to subconfluent cells. Then, adherent cells were pooled with spontaneously detached cells, and incubated in 100 μg/mL propidium iodide (PI), 0.05% NP-40, 10 μg/mL RNase A in PBS at 4°C for ≥3 h. This assay permeabilizes the cells, thus PI stains both live and dead cells. The percentage of apoptotic cells with decreased DNA staining (hypodiploid cells) was counted by flow cytometry using BD CellQuest Software (BD Biosciences, San Jose, CA) [24, 25].

RNA extraction and real-time polymerase chain reaction

For RNA extraction, 200,000 cells were seeded in 60-mm cell culture dishes (BD, Franklin Lakes, NJ) in 10% FCS RPMI overnight. They were rested in serum-free medium for 24 h and p-CS or p-CG was added to subconfluent cells. Three hours later cells were rinsed once with 1 mL of ice cold saline and then total RNA was extracted from cells by the TRI Reagent method (Roche, Mannheim, Germany). This time point was chosen because inflammatory responses in proximal tubular cells are usually observed within this time-frame [26, 27]. One microgram RNA was reverse transcribed with a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Pre-developed primer and probe assays for MCP-1, CXCL16, RANTES, Fn14 and 18S were obtained from Applied Biosystems. A quantitative PCR was performed by 7500 Real-Time PCR System with the Prism 7000 System SDS Software (Applied Biosystems) and RNA expression of different genes was corrected for 18S [26].

Cell surface Fn14 expression

For assessment of Fn14 expression by flow cytometry, 10,000 cells were seeded in 12-well plates (Costar, Cambridge, MA) in 10% FCS RPMI overnight. They were rested in serum-free medium for 24 h. Thereafter, p-CS was added to subconfluent cells and 24 h later they were detached with 2 mM EDTA/1% bovine serum albumin (BSA) in PBS, washed and resuspended in PBS/1% BSA for 4 min. Then, cells
were incubated with a 1 μg/mL anti-Fn14 ITEM4 antibody (eBioscience, San Diego, CA) or an isotype-matched control antibody for 30 min on ice. Cells were washed twice, blocked in PBS/1% BSA for 4 min and incubated with an Alexa488-labelled goat anti-mouse IgG antibody (1/300, Invitrogen, Carlsbad, CA) for 45 min on ice in the dark. Following two additional washes with PBS/1%BSA, cells were resuspended in 1% filtered paraformaldehyde in PBS and analysed by flow cytometry using BD CellQuest Software (BD Biosciences) [28].

**Western blot**

For western blot analysis, 200 000 cells were seeded in 60-mm cell culture dishes (BD, Franklin Lakes, NJ) in 10% FCS RPMI overnight. They were rested in serum-free medium for 24 h. Thereafter, p-CS was added to subconfluent cells and 24 h later, cells were detached with a scraper. Cell samples were homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.1 mM PMSF and 1 μg/mL pepstatin A), then separated by 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. After electrophoresis, samples were transferred to PVDF membranes (Millipore, Billerica, MA), blocked with 5% BSA in PBS/0.5% Tween 20 for 1 h, washed with PBS/Tween and incubated with rabbit polyclonal anti-Fn14 (1:1000, Cell Signaling, Danvers, MA). Anti-Fn14 was diluted in 1% BSA PBS/Tween. Blots were washed with PBS/Tween and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham, Aylesbury, UK). After washing with PBS/Tween blots were developed using the chemiluminescence method (ECL) (Amersham). Blots were then probed with a mouse monoclonal anti-β-actin antibody (1:2000, SIGMA-ALDRICH) and levels of expression were corrected for minor differences in loading [29].

**Statistics**

Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as mean ± SEM. Significance at the P < 0.05 level was assessed by Student’s t test for two groups of data and ANOVA for three or more groups.

**RESULTS**

**p-CS promotes human proximal tubular cell death**

In order to determine the biological activity of uraemic toxins, tubular cells were exposed to different concentrations of p-CS (1 to 500 μg/mL) for 24 h, and the percentage of hypodiploid apoptotic cells was analysed by flow cytometry (Figure 1A). We observed no significant changes in the percentage of apoptotic cells at either 24 h (Figure 1A) or 48 h (not shown), although a trend was observed towards increased cytotoxicity for the higher p-CS concentrations. Since in vivo tubular cells are chronically exposed to uraemic toxins, we redesigned the experiment by adding p-CS or p-CG every other day for a week (Figure 1B). We observed an increment in the apoptotic rate in cells exposed to 100 μg/mL p-CS or higher concentrations. Albumin was toxic to tubular epithelium, as previously described [30], and p-CS did not increase the cytotoxicity over that of albumin (Figure 1B). Apoptotic morphology was confirmed by DAPI staining that disclosed fragmented, bright nuclei among cells exposed to p-CS (Figure 1C). In contrast, p-CG alone was not lethal (Figure 1D) and did not modulate the toxicity of p-CS (not shown).

**p-CS increases expression of the TWEAK receptor Fn14 and TWEAK-induced apoptosis in proximal tubular cells**

HK2 cells were exposed to 100 μg/mL p-CS or 25 μg/mL p-CG for 3 h and then analysed for Fn14 mRNA expression (Figure 2A). This time point was chosen because of prior knowledge of Fn14 biology [23]. Fn14 is encoded by an early response gene and protein expression after cytokine stimulation occurs at this time point. Addition of p-CS increased Fn14 mRNA expression, whereas no changes were observed in p-CG-treated cells. p-CS also increased Fn14 protein expression as assessed either by flow cytometry, which denotes the presence of Fn14 on the cell membrane (Figure 2B and C), or by western blot of whole cell lysates (Figure 2D). Increased Fn14 expression induced by p-CS sensitized cells to death in the presence of TWEAK in short-term, 24-h experiments (Figure 3).

**p-CS is pro-inflammatory in proximal tubular cells**

To study whether uraemic toxins triggered inflammation, we evaluated the effect of these molecules on the expression profile of the chemokines MCP-1, CXCL16 and RANTES. Exposure to 100 μg/mL p-CS significantly increased the gene expression of the three chemokines (Figure 4). In contrast, no differences were observed in p-CG-treated cells. Lower concentrations of p-CS, similar to those found in the circulation of non-dialysis CKD patients (50 μg/mL) [31] increased the mRNA expression of the chemokines MCP-1 and CXCL16 in the presence of albumin (Figure 5A and B). The reason for the albumin requirement is unclear. However, p-CS binds albumin and albumin is present in the tubular lumen of patients with progressive CKD, where it is known to be uptaken and to stress tubular cells [30]. At this concentration, p-CS did not promote RANTES expression (Figure 5C). Although both MCP-1 and RANTES expression by proximal tubular cells is dependent on NFκB [26], transcription of the RANTES gene requires a more persistent activation of this transcription factor since DNA should be made accessible before NFκB activation wanes [27].

**DISCUSSION**

The main findings of our study were that in human proximal tubular epithelial cells p-CS promotes cell death, increases the expression of the TWEAK receptor Fn14 and promotes inflammatory gene expression. These findings suggest that p-CS may contribute to CKD progression by promoting tubular cell injury.
Proximal tubular cells are key cells for normal kidney structure and function. Selective depletion of proximal tubular cells leads to acute kidney injury evolving to kidney fibrosis [32]. Tubular cells are key cells in tubulointerstitial inflammation. In response to an adverse microenvironment, tubular cells are activated to express chemotactic and proinflammatory molecules, including cytokines such as TNF, Fas ligand, TRAIL and TWEAK and chemokines such as MCP-1, RANTES and CXCl16 that decisively contribute to recruit inflammatory cells and to promote kidney injury [26, 33–35]. After a long focus on the glomerulus as a key target of diabetic and non-diabetic nephropathy, recently there has been considerable interest in the role of tubules [36, 37]. Indeed, tubular cells were the first kidney cells described to undergo apoptosis in a high-glucose milieu [38]. In this regard, tubulointerstitial damage correlates better with the outcome of renal function than glomerular injury scores, even for primary glomerulonephritis [39, 40].

$\text{p-CS}$ was cytotoxic to cultured tubular cells, promoting apoptosis. This effect was not observed for $\text{p-CG}$ at the concentrations tested. The increase of tubular cell death and inflammation by $\text{p-CS}$ is relatively mild. However, chronic inflammation and loss of tubular cells in CKD are mild, persistent conditions that lead to slow progression of CKD over months to years. In addition, the present data were obtained by means of subacute experiments with an exposition lasting for only 7 days. A more prolonged in vivo exposure may lead to more persistent and progressive injury. In this regard, other sources of inflammatory cytokines released in the CKD milieu may cooperate with uraemic toxins to promote tubular cell death or inflammation. Thus, the combination of TWEAK and uraemic toxin further increased cell death. Furthermore, tubular cells are targets of additional nephrotoxic agents, including contrast media, non-steroidal anti-inflammatory agents or over-the-counter medications such as paracetamol that may also cooperate in tubular injury [41–43]. The array of cell membrane transporters of tubular cells facilitates entry and accumulation of nephrotoxins in proximal tubular cells. Examples include the accumulation and toxicity of anti-retroviral drugs that cause both acute kidney injury and
CKD such as cidofovir and tenofovir [44, 45]. The same transporters may contribute to accumulation of p-CS or p-CG in tubular cells. Thus, p-CS is actively transported into proximal tubular cells potentially contributing to intracellular accumulation and potential adverse biological effects [46]. In very short-term studies, there was evidence for active uptake of p-CS that was linear for 10–15 min with later stabilization [46]. Serum concentrations of p-CS and p-CG are markedly increased in uraemic patients compared with healthy subjects [9]. The total pre-dialysis concentration of p-CS is higher than that of p-CG. Prolonged in vivo exposure to the very high levels of uraemic toxins found in advanced CKD may result in intracellular accumulation and toxicity of the compounds.

**FIGURE 2:** p-CS increases expression of the TWEAK receptor Fn14 and TWEAK-induced apoptosis in human proximal tubular HK2 cells. (A) HK2 cells were treated with 100 μg/mL p-CS, p-CG 25 μg/mL or their vehicles for 3 h. RNA was reverse transcribed and analysed by quantitative PCR. Fn14 mRNA expression was increased by p-CS but not by p-CG (*P < 0.03 versus vehicle). n = 3. HK2 cells were treated with 100 μg/mL (B) or 200 μg/mL (C) p-CS for 24 h and then collected and stained with anti-Fn14 or control IgG. Membrane expression of Fn14 was measured by flow cytometry. n = 2. (D) HK2 cells were treated with 100 μg/mL p-CS for 24 h. Western blot of total cell protein shows an increased Fn14 expression in p-CS-treated cells (*P < 0.002 versus vehicle). The vehicle used was KCl for p-CS and NH₄Cl for p-CG experiments. n = 3.

**FIGURE 3:** Cooperation of p-CS and cytokines to promote tubular cell death. HK2 cells were treated with 100 ng/mL TWEAK and 200 μg/mL p-CS for 24 h and cell death quantified by flow cytometry of DNA content. Cells were permeabilized and stained with PI to quantify hypodiploid nuclei by flow cytometry (*P < 0.02 versus vehicle, #P < 0.03 versus vehicle + TWEAK) n = 3.
The pro-inflammatory role of these toxins had been previously studied in leukocytes and overall findings are consistent with those observed in tubular cells. p-CS enhanced the percentage of free radical-producing leucocytes at baseline, while p-CG was inert [47]. However, both toxins cooperated in promoting free radical production in leukocytes, while p-CG did not add to the pro-inflammatory or lethal effect of p-CS when tested simultaneously in tubular cells. These toxins may also directly damage the endothelium [10] and promote smooth muscle cell proliferation [48], which may explain their association with cardiovascular disease in the CKD population [47, 49–51]. In this regard, enhancing the removal and/or decreasing the concentration of these protein-bound solutes might slow progression of CKD [52, 53]. Thus, administration of p-CS to mice promoted kidney fibrosis, modulated the epigenetics of the Klotho gene and decreased tubular Klotho expression. In addition, p-CS also decreased Klotho expression in cultured tubular cells [21]. Klotho is a kidney secreted anti-ageing hormone and also a receptor for fibroblast growth factor-23 (FGF-23). Klotho possesses antioxidant, anti-inflammatory, antifibrotic and cytoprotective properties [54–56]. Klotho expression by kidney tubular cells had been previously shown to be downregulated by inflammatory cytokines in cell culture and in vivo [57]. Specifically, the inflammatory cytokine TWEAK downregulated Klotho expression through NFκB binding to the Klotho promoter and through epigenetic modulation of the gene. p-CS upregulation of the TWEAK receptor Fn14 could contribute to the downregulation of Klotho expression by uraemic toxins in vivo. In this regard, the TWEAK/Fn14 system is regulated mainly by levels of expression of the Fn14 receptor [58] and has been shown to be
a key promoter of tubular cell death and inflammation in the course of nephrotoxic AKI [26]. More recently, p-CS was shown to cause oxidative stress and to promote a fibrogenic response in tubular epithelium [59].

Another uraemic toxin, indoxyl sulphate, has adverse effects on tubular cells that include oxidative stress, inhibition of cell proliferation, apoptosis, promotion of inflammation and secretion of profibrotic factors such as TGF-β1 and epithelial-to-mesenchymal transition of renal tubular cells [60–66]. Since both indoxyl sulphate and p-CS are present in the uraemic milieu it is conceivable that they may interact or cooperate to promote kidney injury. In this regard both indoxyl sulphate and p-CS have been suggested to be transported into proximal tubular cells by organic anion transporters [46, 62, 67], although this view has been disputed [68].

The clinical relevance of these findings rests on the observation that clinically relevant concentrations of p-CS induced proximal tubular cell stress, manifested as an inflammatory response or death. At p-CS concentrations found in non-dialysis CKD patients [5, 31, 47], a proinflammatory response was observed in the presence of albumin. Albuminuria is a frequent feature of progressive CKD, has tubulotoxic actions and may accelerate progression of kidney disease [30]. At p-CS concentrations found in dialysis patients, a proapoptotic response was observed. The relevance of tubular cell loss in dialysis patients may be disputed. However, tubular cells have an endocrine function that includes activation of vitamin D and secretion of Klotho. Maintenance of this endocrine function may be beneficial for dialysis patients. In fact, residual renal function preservation is associated with improved outcomes in dialysis [69].

The concentration ranges studied were 1–500 μg/mL p-CS and 25 or 50 μg/mL for p-CG. The total serum levels of p-CS and p-CG in healthy volunteers were 2.87 ± 1.71 and 0.35 ± 0.03 μg/mL and in the haemodialysis population reached up to 105 and 24 μg/mL, respectively [9, 70]. These values correspond to free p-CS 16.3 μg/mL and free p-CG 22.4 μg/mL. There is very little information on p-CS values in patients with non-dialysis CKD. An analysis of 10 CKD Stage 5 patients disclosed mean (SD) p-CS levels of 29 (17) μg/mL [31]. That is, 16% of patients had values >46 μg/mL. Additional authors described p-CS levels 2.5-fold higher in CKD Stage 1–5 progressors versus non-progressors [5]. The interaction between albumin and p-CS may take several hypothetical forms that may impact the in vivo effects of p-CS on proximal tubular cells. p-CS will bind to albumin decreasing the available free p-CS fraction. However, uptake of albumin by proximal tubular cells may facilitate p-CS entry into the cells, especially in the presence of albuminuria. Finally, there might be additional interactions between the biological activities on proximal tubular cells of albumin and p-CS.

In conclusion, p-CS has proapoptotic and pro-inflammatory effects on human proximal tubular epithelial cells, which may cooperate with other stressors such as the inflammatory cytokine TWEAK to promote progression of CKD.

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CONFLICT OF INTEREST STATEMENT

None declared.

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