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p-Cresyl sulfate causes renal tubular cell damage by inducing oxidative stress by activation of NADPH oxidase

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The accumulation of *p*-cresyl sulfate (PCS), a uremic toxin, is associated with the mortality rate of chronic kidney disease patients; however, the biological functions and the mechanism of its action remain largely unknown. Here we determine whether PCS enhances the production of reactive oxygen species (ROS) in renal tubular cells resulting in cytotoxicity. PCS exhibited pro-oxidant properties in human tubular epithelial cells by enhancing NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) activity. PCS also upregulated mRNA levels of inflammatory cytokines and active TGF-β1 protein secretion associated with renal fibrosis. Knockdown of $p22^{phox}$ or Nox4 expression suppressed the effect of PCS, underlining the importance of NADPH oxidase activation on its mechanism of action. PCS also reduced cell viability by increasing ROS production. The toxicity of PCS was largely suppressed in the presence of probenecid, an organic acid transport inhibitor. Administration of PCS for 4 weeks caused significant renal tubular damage in 5/6-nephrectomized rats by enhancing oxidative stress. Thus, the renal toxicity of PCS is attributed to its intracellular accumulation, leading to both increased NADPH oxidase activity and ROS production, which, in turn, triggers induction of inflammatory cytokines involved in renal fibrosis. This mechanism is similar to that for the renal toxicity of indoxyl sulfate.

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In chronic kidney disease (CKD), metabolic changes and an impaired urinary excretion of metabolites lead to the accumulation of uremic toxins in the body.[1] Indoxyl sulfate (IS) is one of the most extensively studied uremic toxins. Indeed, the mechanism by which IS induces its toxicity has now been clarified.[2–5] It seems likely that the intracellular accumulation of IS *via* organic anion transporters (OATs) and increased oxidative stress by reactive oxygen species (ROS) production have a key role in the toxicity of IS.[6–8]

Along with IS, *p*-cresol is also a well-characterized protein-bound uremic toxin.[9] *P*-cresol is known to exert a wide variety of biological activities, including endothelial dysfunction[9] and the suppression of respiratory burst action in the blood, and might also mediate the progression of CKD.[10–12] Recent advances in analytical techniques have revealed that most of the *p*-cresol synthesized by enteric bacteria undergoes conjugation with sulfate *via* the action of sulfotransferases in the submucosal tissue of the small intestine.[13,14] Thus, *p*-cresol is present in the plasma in the form of *p*-cresyl sulfate (PCS). More recently, clinical studies have shown that the accumulation of PCS is related to the progression of CKD. Indeed, the buildup of PCS appears to be associated with the mortality rate for CKD patients. As such, PCS could be a good predictor for the prognosis of CKD patients.[15–17]

For this reason, the biological activities of PCS, rather than of *p*-cresol, have been the subject of considerable research interest, especially the toxicity of PCS in the kidney and organs associated with complications of CKD.

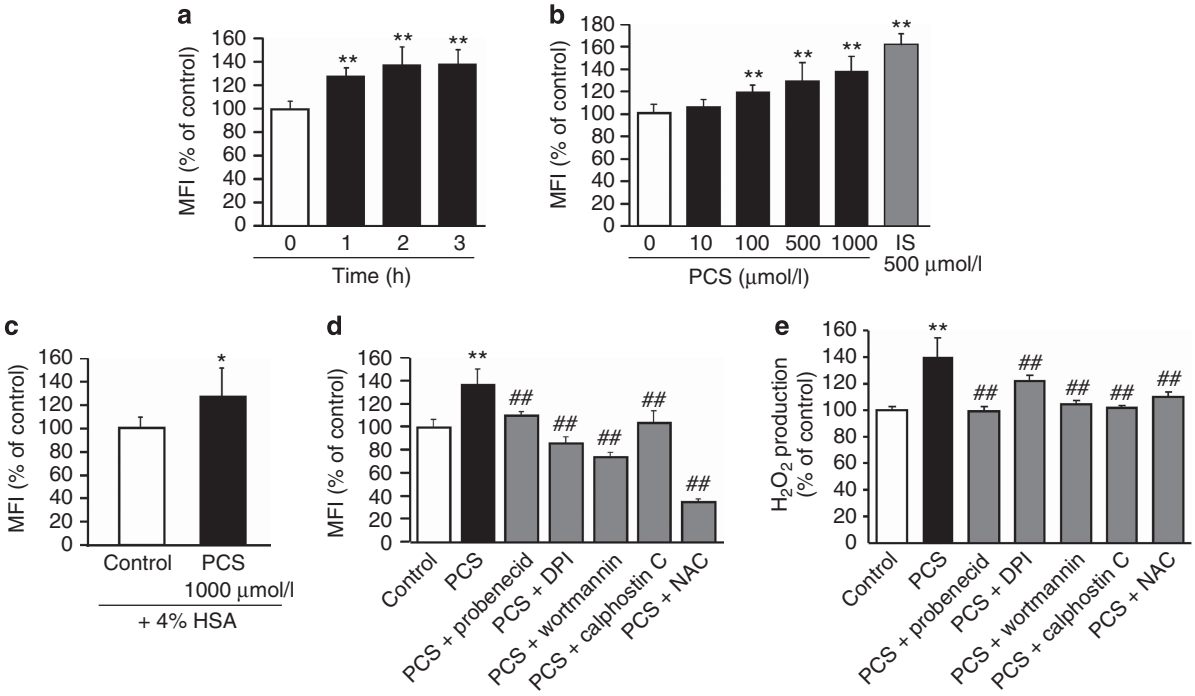

Figure 1 | Effect of *p*-cresyl sulfate (PCS) on reactive oxygen species (ROS) production in HK-2 cells. (**a**) Time course and (**b**) concentration dependency (for 3 h incubation) for PCS-induced ROS production in HK-2 cells. (**c**) Effect of 4% human serum albumin (HSA) on PCS-induced ROS production in HK-2 cells. (**d**) Effect of an OAT inhibitor (1000 μmol/l probenecid), NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) inhibitor (50 μmol/l DPI), PI3K inhibitor (5 μmol/l wortmannin), protein kinase C inhibitor (1 μmol/l calphostin C), and *N*-acetyl-L-cysteine (NAC; 1000 μmol/l) on PCS-induced ROS production in HK-2 cells. (**e**) Extracellular H_2O_2 was determined in the absence or presence of 1000 μmol/l probenecid, 50 μmol/l diphenylene iodonium (DPI), 5 μmol/l wortmannin, 1 μmol/l calphostin C, and 1000 μmol/l NAC. Results are expressed as percentages of fluorescence intensity compared with control (0 μmol/l PCS) (mean ± s.d., $n = 6$, $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. PCS). MFI, mean fluorescent intensity.

In contrast to *p*-cresol, the biological activity of PCS is largely unknown. Scheper *et al.*[12] recently reported that PCS, unlike *p*-cresol, is a potent activator of leukocytes. This finding indicates that PCS and *p*-cresol possess different biological activities as a result of the conjugation. However, the precise mechanism by which PCS exerts its toxicity remains largely unknown. Here, we hypothesized that PCS accumulates in target cells and induces oxidative stress, thereby exhibiting cytotoxicity. In fact, our previous investigations with rat renal cortical slices and HK-2 cells as a model of human proximal tubular cells showed that PCS serves as a substrate for OATs and accumulates in the kidney *via* these transporters.[18]

In the present study, we examined the potential of PCS to produce ROS using an HK-2 cell line to characterize its renal toxicity by comparison with IS. We also examined the influence of oxidative stress induced by PCS on inflammatory cytokine production and cell viability. Moreover, to further confirm PCS action *in vivo*, PCS was administrated to 5/6-nephrectomized rats and its renal toxicity was evaluated.

RESULTS

Effect of PCS on ROS production in HK-2 cells

To determine whether PCS induces oxidative stress in HK-2 cells, we examined the effect of PCS on ROS production in HK-2 cells. Experiments were performed under identical conditions using IS as a positive control. The findings indicated that PCS significantly increased ROS production in a time-dependent manner reaching a maximum after 3 h (Figure 1a) and that this increase was concentration dependent (10, 100, 500, and 1000 μM) (Figure 1b). As previously reported, IS also induced ROS production in HK-2 cells to an extent comparable to that of PCS. Because PCS is tightly bound to albumin in the blood (>90%),[19] the effect of PCS on ROS production was also examined in the presence of 4% human serum albumin (HSA). The enhancement of ROS production in HK-2 cells by PCS was slightly reduced in the presence of 4% HSA (90% of ROS production in the absence of HSA; see Figure 1c).

Next, we investigated the mechanism of the pro-oxidant properties of PCS. This was done by conducting the same set of experiments in the presence of various inhibitors, such as a transporter protein inhibitor (probenecid), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) inhibitor (diphenylene iodonium (DPI)), PI3K inhibitor (wortmannin), and a protein kinase C (PKC) inhibitor (calphostin C). As shown in Figure 1d, ROS induced by PCS was significantly inhibited by all of these inhibitors, suggesting the contribution of the NADPH oxidase system. If NADPH oxidase is involved in PCS-induced ROS production, extracellular hydrogen peroxide (H_2O_2) should be enhanced through superoxide generation by NADPH oxidase. Therefore, we estimated the production of extracellular H_2O_2 by Amplex