P-cresylsulphate, the main \textit{in vivo} metabolite of \textit{p}-cresol, activates leucocyte free radical production

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Abstract

\textbf{Background.} Chronic renal insufficiency is associated with the retention of solutes normally excreted by healthy kidneys. \textit{p}-cresol, a prototype protein-bound uraemic retention solute, has been shown to exert toxic effects \textit{in vitro}. Recently, however, it has been demonstrated that \textit{p}-cresol in the human body is conjugated, with \textit{p}-cresylsulphate as the main metabolite.

\textbf{Methods.} The present study evaluates the effect of \textit{p}-cresylsulphate on the respiratory burst activity of leucocytes.

\textbf{Results.} \textit{P}-cresylsulphate significantly increased the percentage of leucocytes displaying oxidative burst activity at baseline. Oxidative burst activity of stimulated leucocytes was however not affected. In contrast, \textit{p}-cresol had no effect on the leucocytes at baseline, but inhibited leucocytes burst activity after stimulation.

\textbf{Conclusion.} The present study demonstrates, for the first time, that \textit{p}-cresylsulphate, the main \textit{in vivo} metabolite of \textit{p}-cresol, has a pro-inflammatory effect on unstimulated leucocytes. This effect could contribute to the propensity to vascular disease in the uraemic population.

\textbf{Keywords:} immune response; leucocytes; oxidative burst activity; uraemic toxins; vascular disease

Introduction

Chronic renal failure is characterized by the retention of solutes that are normally cleared into the urine. These solutes may deteriorate biochemical and physiological functions defining the uraemic syndrome [1,2]. Atherosclerosis remains the main cause of death among patients with stage 3–5 kidney disease [3] and may be associated with the baseline inflammatory status of many of these patients [4–6]. In contrast, their blunted immune response after stimulation is related to an increased susceptibility to infection [7,8].

\textit{P}-cresol, a volatile phenol with a molecular weight of 108.1 Da, is strongly protein-bound. It exerts various toxic effects \textit{in vitro}. \textit{P}-cresol decreases the response of activated polymorphonuclears [9]. It inhibits platelet activating factor synthesis by human adherent monocytes [10] and decreases the endothelial cell response to inflammatory cytokines [11]. Total and/or free \textit{p}-cresol concentrations have repeatedly been linked to parameters of clinical condition and outcome [12–14].

\textit{P}-cresol emanates from the metabolism of the amino acids tyrosine and phenylalanine by the intestinal flora. These amino acids are generated from nutritional proteins and are metabolized into 4-hydroxyphenylacetic acid which is decarboxylated to \textit{p}-cresol [15]. During passage through the intestinal mucosa, a cytosolic sulphotransferase has the potential to metabolize \textit{p}-cresol resulting in \textit{p}-cresylsulphate [16].

Two recent publications showed that unconjugated \textit{p}-cresol is not detectable in normal and uraemic human plasma [17,18]. According to these studies, the majority of the intestinally generated \textit{p}-cresol appears in the circulation as \textit{p}-cresylsulphate, whereas a minor fraction is glucuronidated. The earlier misinterpretation, hypothesizing that \textit{p}-cresol was present as such, is attributable to sample preparation using strong acidification for deproteinization, resulting in the hydrolysis of the conjugates of \textit{p}-cresol. Application of non-acidic deproteinization strategies makes detection of the sulphated compound possible [18], which is entirely desintegrated with acidification.

The present study evaluates, for the first time, biological toxicity of \textit{p}-cresylsulphate in comparison with \textit{p}-cresol. In this study, whole blood was incubated in the presence of \textit{p}-cresylsulphate and an increase of free radical production by unstimulated leucocytes was observed.
Materials and methods

Sample collection

After informed consent, blood from healthy donors was collected into sodium heparin Vacutainer™ tubes (Becton Dickinson, San Jose, CA, USA). Persons who smoked or had quit smoking for less than 3 months before the study, those who had an infection, were pregnant or on medication were excluded from the study. The present study was approved by the local Ethics Committee.

Reagents

A p-cresol solution of 5000 µg/ml in 100% methanol was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). P-cresylsulphate was synthesized as described by Feigenbaum and Neuberg [19], resulting in the potassium salt of the compound.

Experimental setup

The p-cresol stock solution was diluted in saline, a 0.9% sodium chloride solution (Baxter, Lessines, Belgium), to a 10 times stock solution in order to test the compound at a final concentration of 40.7 mg/l, its assumed maximal concentration (Cmax) reported in uraemic patients [20]. Since p-cresol is dissolved in absolute methanol, a control sample containing an equal amount of the solvent (0.81%) (Acros Organics, Geel, Belgium) was included as a solvent control next to a saline control.

Data on uraemic concentrations of p-cresylsulphate are up to now rather scarce. Individual concentrations are described only by Martinez et al. [18]. Based on their Curaemic value of 50 mg/l, a concentration which in the meanwhile is confirmed by our own laboratory (unpublished data), the Cmax was calculated as follows: The ratio maximal reported uraemic concentration/mean uraemic concentration (Cmax/Curaemic) for p-cresol is 2 (=40.7/20.1 mg/l), therefore, a Cmax was chosen of 100 mg/l for p-cresylsulphate which is twice the Curaemic concentration (50 mg/l). Because p-cresylsulphate was synthesized as a potassium salt, 121 mg/l of this salt was dissolved in saline. A solution of 0.53 mmol/l KCl (Sigma-Aldrich Co, St Louis, MO, USA) in saline, which was chosen to equal the potassium concentration in the K-salt of p-cresylsulphate, was included as a salt control next to a saline control.

Oxidative burst

Heparinized whole blood was incubated for 10 min at 37°C with saline (control), p-cresol, p-cresylsulphate or their respective solvent control 0.81% methanol (MeOH) and salt control 0.53 mmol/l KCl (KCl) in Falcon® polystyrene tubes (Becton Dickinson). To evaluate the effect of the solutes on the oxidative burst activity of the leucocytes, the Bursttest (Phagoburst®) (Orpegen Pharma, Heidelberg, Germany) was applied according to the manufacturer’s guidelines. The burst activity in leucocytes was evaluated at baseline and after stimulation with N-formyl-methionine-leucine-phenylalanine (fMLP) as a moderate stimulus and Escherichia coli (E. coli) or phorbol-12-myristate-13-acetate (PMA) as strong stimuli. By the conversion of the fluorogenic substrate dihydrorhodamine into rhodamine, the generation of the reactive oxygen metabolites superoxide anion, hydrogen peroxide and hypochlorous acid is monitored.

Within 30 min, the samples were analyzed using a FACSscan® flow cytometer (Becton Dickinson). For the baseline status and after fMLP stimulation, the percentage (%) of rhodamine positive cells was considered. Since the large majority of cells after stimulation with E. coli or PMA were rhodamine positive (>85%), for the latter stimuli, the mean fluorescence intensity (MFI) per cell was evaluated as a parameter for the oxidative burst activity. A marker was placed to exclude background fluorescence.

Endotoxin concentration

All experimental solutions causing a stimulation of the oxidative burst were checked for endotoxin contamination, by means of the Limulus Amebocyte Lysate (LAL) QCL-1000-test®, a quantitative kinetic and chromogenic assay (Cambrex Bio Science, Walkersville, MD, USA). The detection limit of this assay is 0.005 endotoxin units/ml.

Viability

The viability of leucocytes, after a 10-min incubation period at 37°C in the presence of the different uraemic solutes, was tested, using the DNA staining solution 7-amino-actinomycin D (7-AAD) and the IOTest® Lysing Solution (Beckman Coulter, Fullerton, California, USA) according to the manufacturer’s guidelines.

Statistical analysis

Data are expressed as mean ± SD. Statistics were performed using a non-parametric paired Wilcoxon test. A P-value of <0.05 was considered significant.

Results

Endotoxin contamination and viability

Since all solutions tested had an endotoxin concentration below 0.005 EU/ml, none of the observed stimulations was attributable to endotoxin contamination.

Cell viability in solutions containing p-cresol exceeded 95%, meaning that the inhibitory effects described subsequently were not due to an increased amount of dead cells. In addition, also p-cresylsulphate did not influence the cell viability.

Effect of p-cresylsulphate in comparison with p-cresol on baseline oxidative burst activity of leucocytes

At baseline, between 3 and 5% of leucocytes were found to produce free radicals as illustrated in Figure 1.
KCl, the salt control of p-cresylsulphate, had no effect on the percentage of rhodamine positive cells in comparison with saline alone. P-cresylsulphate enhanced the percentage of free radical producing leucocytes compared with KCl as illustrated in Figure 1A. A significant rise in the percentage of rhodamine positive cells was observed for monocytes and lymphocytes (P < 0.02, n = 10). For granulocytes, a similar trend was observed, but it was not significant. MeOH, the solvent control of p-cresol, caused a decrease in the percentage of free radical producing cells vs saline (Figure 1B), which was only significant for granulocytes (P < 0.01, n = 8). In the presence of p-cresol no effect was observed vs MeOH in the three cell types; hence the observed inhibitory effect of p-cresol vs saline is very likely only attributable to the presence of the solvent, rather than to p-cresol per se.

Since the oxidative burst activity is a representative marker for the inflammatory status of leucocytes, and since a baseline activation of leucocytes has been related to vascular disease [4–6], the present data suggest that p-cresylsulphate is a potential contributor to the vascular damage in chronic kidney disease.

**Effect of p-cresylsulphate in comparison with p-cresol on the stimulated oxidative burst activity of leucocytes**

As shown in Table 1, after stimulation with fMLP, *E. coli* or PMA, in the presence of p-cresylsulphate no significant effect on the leucocyte oxidative burst activity was observed.

While p-cresol did not affect the oxidative burst activity of unstimulated leucocytes (monocytes, lymphocytes and granulocytes), a significant inhibition vs the solvent control, MeOH, was observed for the three types of leucocytes after stimulation with fMLP, *E. coli* and PMA.

Hence, our data suggest that the previously described inhibitory impact of genuine p-cresol on leucocyte function [9] is neutralized by its conjugation to p-cresylsulphate, resulting in a net detoxification effect.

**Discussion**

In this study, the biological effect of p-cresylsulphate, a metabolite of p-cresol, was evaluated for the first time and compared with unconjugated p-cresol. The oxidative burst activity of leucocytes was evaluated at baseline and after stimulation with fMLP, *E. coli* and PMA in the presence of p-cresylsulphate.

P-cresylsulphate enhanced the percentage of free radical producing leucocytes at baseline. This suggests that p-cresylsulphate could contribute to the propensity to vascular disease in chronic renal failure.

It is of note that the increase of leucocyte activity by p-cresylsulphate is relatively mild. However, chronic inflammation as a typical feature of chronic kidney disease, associated with C-reactive protein (CRP) values even in the high normal range [21], and can likewise be considered as being mild. In addition, the present data were obtained by means of acute experiments with an exposition lasting for only 10 min. Since *in vivo* exposure, on the contrary, is continuous, the damaging impact may be more persistent.

In the baseline condition, the impact of p-cresylsulphate is obviously more relevant than that of p-cresol, which was inert at baseline. The question...
arises whether in clinical reality, the concentrations of p-cresyl sulphate can be supposed to correlate to the previously measured p-cresol values as determined by acidification methods; that this is indeed the case, is suggested in an indirect way by de Looor et al. [17] and confirmed in a direct way by our own group (non-published observations). Hence, it is very possible, as explained subsequently, that the previously found relationship of p-cresol with the clinical condition [12–14] remains relevant, but that these correlations are to a large extent attributable to p-cresyl sulphate, rather than to p-cresol per se; this assumption necessitates further evaluation, however.

As previously described and confirmed in the present study, the oxidative burst activity in stimulated leucocytes was inhibited after incubation with p-cresol [9]. P-cresyl sulphate on the other hand did not have any significant effect on the activated leucocytes. Nevertheless, one should consider the presence in several cell systems of sulphatases, e.g. in leucocytes, which have the potential to deconjugate sulphates. Human neutrophils were demonstrated to be a rich source of arylsulphatases (AS), which catalyse the hydrolysis of aromatic sulphate esters [22]. Türkmen et al. [23] showed elevated AS activity in leucocytes isolated from women with breast cancer and Oner et al. [24] suggested elevated AS activity in leucocytes from healthy women. In case of acute alcohol intoxication, a significant elevation in the plasma of AS and another lysosomal enzyme β-glucuronidase was detected [25].

Because of this AS activity in leucocytes, and since these cells are known to secrete lysosomal enzymes upon stimulation [26], chronically activated leucocytes in uraemia could be involved in the desulphation of p-cresyl sulphate to p-cresol in vivo. In this way, the generation of p-cresol in the immediate surroundings of leucocytes could still impair the response to infection of the uraemic patient.

The uraemic solute indoxyl sulphate, another sulphated conjugate, inhibits endothelial cell proliferation and wound repair in vitro [27] and is supposed to contribute to the endothelial dysfunction in chronic kidney disease. In a study by Niwa and Ise [28], indoxyl sulphate was shown to accelerate the in vivo progression of chronic renal failure in rats. Miyazaki et al. [29] demonstrated that the addition of indoxyl sulphate to cultured proximal tubular cells significantly increased the synthesis of transforming growth factor-β1. This points to the fact that p-cresyl sulphate is not the only sulphated conjugate of a known uraemic retention solute to exert toxicity, and that in the future even more biological effects than the one described in the present study will be revealed.

In conclusion, the present study demonstrates for the first time that the in vivo circulating metabolite of p-cresol, p-cresyl sulphate, has a pro-inflammatory effect as evaluated by the increased oxidative burst activity of leucocytes at baseline, and that it, therefore, may contribute to the propensity to vascular damage in renal patients.

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Conflict of interest statement. None declared.

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