The uremic solute \( p \)-cresol decreases leukocyte transendothelial migration \textit{in vitro}

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Abstract

Chronic renal failure (CRF) patients display an immunodeficiency state, and uremic solutes that accumulate during CRF may be involved in this immunodeficiency. In this study, we examined whether the uremic solute \( \text{para-cresol (} p \)-cresol\), at concentrations similar to those found in patients, alters leukocyte transmigration \textit{in vitro}. We found that \( p \)-cresol significantly inhibited monocyte THP-1 cell line and PBMCs transmigration across IL-1\( \beta \)-stimulated human umbilical vein endothelial cell (HUVEC) in a static two-compartment model. This inhibitory effect of \( p \)-cresol persisted in the presence of a physiologic concentration of human serum albumin. In order to investigate the mechanism involved, expression of endothelial chemokines, fractalkine, monocyte chemoattractant protein 1 (MCP-1) and IL-8 and membrane expression of junctional adhesion molecule A (JAM-A or JAM-1) were studied. We found that \( p \)-cresol decreased mRNA expression of the chemokine fractalkine in IL-1\( \beta \)-stimulated HUVEC, without modifying mRNA expression of MCP-1 and IL-8. In addition, \( p \)-cresol decreased IL-1\( \beta \)-induced expression of membrane-bound and soluble forms of fractalkine and impaired the membrane expression of JAM-A. Taken together, these results suggest that \( p \)-cresol, by impairing leukocyte transendothelial migration, plays a role in the immune dysfunction of uremic patients.

Introduction

Infectious diseases are among the major morbid events in chronic renal failure (CRF) patients (1, 2). These patients clearly display immune dysfunction, illustrated by their high rate of bacterial infections and impaired vaccine response (3, 4). Moreover, monocytes and neutrophils from uremic patients exhibit alterations of their phagocytic and chemotactic functions (5, 6). The accumulation of some uremic solutes may participate in this defective immune response. Indeed, uremic biological fluids alter immune functions \textit{in vitro} (7, 8). More specifically, it appears that the protein-bound uremic solute \( \text{para-cresol (} 4 \)-methylphenol, \( p \)-cresol\) plays a role in the immunodeficiency of CRF patients. \textit{In vitro}, \( p \)-cresol depresses phagocyte functional capacity (9) and inhibits the release of platelet-activating factor by macrophages (10). In addition, De Smet \textit{et al.} (11) showed a link between high concentrations of plasma-free \( p \)-cresol and hospitalization rates for infectious diseases in CRF patients.

Leukocyte transendothelial migration (or transmigration) is a key event in immune response, since it concerns both innate and adaptative immunity. Leukocyte transmigration is a multi-step process. Leukocytes roll along the vessel wall to the sites of inflammation and adhere firmly to the endothelium (12). Adherent leukocytes then crawl through endothelial junctions and migrate to infectious sites, in the final step of transendothelial migration called diapedesis (12, 13). During all these steps, endothelial cells activated by pro-inflammatory cytokines produce chemotactic factors such as monocyte chemoattractant protein 1 (MCP-1) and IL-8, in order to induce chemotaxis and activation of leukocytes (14, 15). Activated endothelial cells also express fractalkine (CX3CL1), which acts both as an adhesion molecule for leukocytes through a membrane-bound form and as a chemokine through a soluble form (16–18). Among endothelial adhesion molecules involved in transmigration, junctional adhesion molecule A (JAM-A) plays a key role. Indeed, it has been demonstrated to interact with its leukocyte counterpart lymphocyte function-associated antigen 1 to participate in the driving of leukocyte along the endothelial junctions (13).
We showed in a previous study that p-cresol inhibits cytokine-induced expression of adhesion molecules on endothelial cells and inhibits monocyte adhesion to stimulated endothelium (19). Thus, we investigated here whether p-cresol could impair leukocyte transendothelial migration. To study the mechanisms involved, we analyzed the effect of p-cresol on the expression of MCP-1, IL-8, fractalkine and JAM-A, which are crucial for all steps of leukocyte transmigration.

Methods

Reagents and antibodies

p-Cresol, human fibronectin, BSA, sodium azide and 70-kDa FITC-conjugated dextran (FITC–dextran) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Methanol was purchased from Carlo Erba (Milano, Italy) and endothelial growth medium 2 (EGM-2) medium from Clonetics Biowhittaker (Verviers, Belgium). PBS, RPMI 1640 medium with glutamax 1, trypsin–EDTA solution, gelatin, HEPES buffer, trypan blue and penicillin-streptomycin were obtained from Invitrogen (Cergy-Pontoise, France). Fetal bovine serum (FBS) was from Dominique Dutscher (Brumath, France), and human serum albumin (HSA) solution 20% was from LFB (Courtaboeuf, France). Lymphocyte separation medium was from Eurobio (Les Ulis, France). IL-1β was purchased from Roche Molecular Biochemicals (Meylan, France), and Calcein-AM was from Molecular Probes (Eugene, OR, USA). mAb against fractalkine (CX3CCL1, clone 51637, IgG1 isotype) was from R&D Systems (Les Ulis, France), mAb against JAM-A (clone BV16) was from Cell Sciences (Canton, MA, USA), and isotype-matched mAb of irrelevant specificity IgG1 (clone 679.1MC7) was from Beckman Coulter Immunotech. FITC-conjugated sheep anti-mouse Ig F(ab′)2 fraction was purchased from Eurobio. QIFIKIT beads were from Dako (Trappes, France). Cytotoxicity detection kit was from Beckmann Coulter Immunotech.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cord vein by collagenase digestion as previously described (20). Cells were seeded on gelatin-coated culture plates and grown in EGM-2 medium under standard cell culture conditions (humidified atmosphere, 5% CO₂, 37°C). Cells were then detached with a 0.05% trypsin–0.02% EDTA solution and subcultured to the second passage until they reached confluence.

The THP-1 cell line was maintained in RPMI 1640 medium with glutamax 1 supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, under standard cell culture conditions. PBMCs were isolated from heparinized venous whole blood of healthy volunteers, by density gradient centrifugation over lymphocyte separation medium within 2 h.

Treatment with p-cresol and cell viability study

p-Cresol was diluted from a stock solution at 500 mg ml⁻¹ in methanol. Cells were incubated in EGM-2 medium containing different concentrations of p-cresol or 0.01% methanol (control medium), in the presence of IL-1β. The condition ‘IL-1β’ includes IL-1β and 0.01% methanol since stock solution of p-cresol was prepared in methanol. The concentrations of p-cresol were 50 μg ml⁻¹ (maximal uremic concentration), 25 and 10 μg ml⁻¹ (mean uremic concentration) and 1 μg ml⁻¹ (normal concentration) (21). Since p-cresol is protein bound, the medium was supplemented with 4% of human albumin (HSA) in some experiments.

HUVECs, THP-1 and PBMC cell viability was monitored by trypan blue exclusion. Apoptosis was studied by flow cytometry after double staining with annexin-V and propidium iodide. Viability was similar in the different experimental conditions and was always >85%. No induction of apoptosis was detected (not shown).

Transendothelial migration assay

Transmigration static assay was performed as described (22). HUVECs were seeded at 60 000 cells per well on fibronectin-coated membrane inserts (porosity 3 μm) of a 24-multiwell double-chamber system (high throughput screening Multiwell Insert System, BD Biosciences, Le pont de Clai, France) and allowed to reach confluence. Confluent endothelial cell monolayers were incubated for 24 h with control medium or medium containing different concentrations of p-cresol, with IL-1β (50 U ml⁻¹), in both compartments. Then, HUVECs were washed with RPMI, and THP-1 (6 × 10⁵ cells per well) were added in the upper chamber, in medium containing p-cresol. THP-1 were allowed to migrate through the endothelial monolayer overnight. Experiments were also performed in media supplemented with 4% HSA. All experiments were run in triplicate. Thereafter, migrated THP-1 present in the lower chamber were incubated with Calcein-AM (10 μM) for 30 min at 37°C. Fluorescence intensity was measured using a Cytofluor® Series 4000 Fluorescence multiwell plate reader (PerSeptive Biosystem, Framingham, MA, USA). The excitation wavelength was 485 nm and the emission wavelength was 530 nm. Absolute cell counts were determined by comparison with dilution series of Calcein-AM-labeled THP-1 cells, made to obtain a linear relationship between fluorescence intensity (arbitrary units) and the corresponding cell count. For experiments in the presence of HSA, the number of transmigrated THP-1 was counted using a cell-counting chamber of Neubauer because of the non-specific fluorescence of Calcein-AM in the presence of HSA, which could be attributable to an esterase-like activity of HSA (23).

The transendothelial migration assay was also performed using PBMCs from healthy donors added in the inserts (porosity 8 μm) of the double-chamber system in the same experimental conditions. In each transmigration assay, endothelial monolayer permeability, assessed using FITC–dextran (24), was comparable in resting cells.

Comparative quantification of fractalkine, MCP-1 and IL-8 mRNA levels

mRNA expression of fractalkine, MCP-1 and IL-8 was studied by reverse transcription (RT) and comparative PCR. HUVECs were incubated 12 h with medium containing methanol (control medium) or p-cresol at maximal uremic concentration.
(50 μg ml⁻¹), with or without stimulation by IL-1β (50 U ml⁻¹). Total RNA was extracted from the HUVECs by an RNAeasy mini-kit (Qiagen, Courtaboeuf, France) according to manufacturer’s instructions, and DNA was digested with DNase (RNase-Free DNase Set, Qiagen) to secure complete DNA removal. Total RNA concentrations were determined by spectrophotometry at 260-nm wavelength. The samples were stored at −80°C for further RT-PCR. RT using random primers was performed on 1 μg of total RNA of each sample using the Startscript First-Strand Synthesis System (Stratagen Europe, Amsterdam, Netherlands), followed by PCR on 20 ng of cDNA using Full Velocity™ SYBR Green QPCR Master Mix (Stratagen Europe). RT of fractalkine mRNA was performed with a specific primer (5'-TGTGCTTCTGCTCCAAG-3'). The housekeeping genes GAPDH and hypoxanthine phosphoribosyl transferase (HPRT) give the same results for normalization of the target gene values. The results shown in this study were those normalized with GAPDH. The sequences of primers were the following: MCP-1—R (5'-CCCCAGTCACCTGCTTAT-3'), MCP-1—F (5'-TGGAGTCTCGAACCACCTTC-3'), IL-8—R (5'-GTGCGATTGTGCGAACAGGT-3'), IL-8—F (5'-CTCTCGACCCAGTTTCTTCTT-3'), fractalkine—F (5'-TCCAAGATGATGCCTGGTGTT-3'), fractalkine—R (5'-TCTGGCCACTGACTGTCCTG-3'), GAPDH—F (5'-GGATTATACTGCTGATGGGAAGC-3'), HPRT—F (5'-GGATTATACTGCTGACCAAGGAAAGC-3').

**Flow cytometry analysis of fractalkine and JAM-A membrane expression**

HUVECs were incubated for 24 h in medium containing methanol (control medium) or p-cresol, in the presence of IL-1β (50 U ml⁻¹). HUVECs were detached with a pre-warmed 0.05% trypsin–0.02% EDTA solution. Cells were washed with PBS–0.1% BSA–0.1% sodium azide and incubated with 50 μl (50 μg ml⁻¹) of anti-fractalkine mAb, anti-JAM-A mAb (50 μg ml⁻¹) or with irrelevant isotype-matched control mAb (50 μg ml⁻¹) for 60 min at 4°C. After being washed, cells were labeled with 100 μl (10 μg ml⁻¹) of FITC-conjugated sheep anti-mouse Ig F(ab')₂ fraction for 45 min at 4°C. Cells were washed and immediately analyzed using an FC500 flow cytometer (Beckman-Coulter, Roissy, France). Mean fluorescence intensity was calculated by the CXP software (Beckman-Coulter) and expressed in arbitrary units. The fluorescence intensity obtained for each antibody corrected for isotype control values was converted into number of mAb-binding sites per cell using QIFIKIT® standard beads (19, 25).

**Measurement of soluble fractalkine in HUVEC supernatants**

After a 24-h incubation of confluent HUVECs with medium containing IL-1β (50 U ml⁻¹) and p-cresol, supernatants were collected, centrifuged at 2000 r.p.m. for 10 min and frozen at −80°C for subsequent use. Fractalkine concentrations in HUVEC supernatants were determined by ELISA with a commercial kit (Fractalkine Duoset, R&D Systems Europe) according to the manufacturer's instructions.

**Statistical analysis**

Statistical analysis was performed with the Prism® software (GraphPad Software Inc, San Diego, CA, USA). Determination of significant differences was performed using a non-parametric analysis with the Friedman test, followed by the Dunns test or Student’s test, depending on assays. A P value lower than 0.05 was considered significant. Data are expressed as mean ± SEM.

**Results**

*p-Cresol decreased THP-1 transendothelial migration**

The ability of p-cresol to alter transmigration of THP-1 monocytes was tested in an *in vitro* static assay of transendothelial migration. The stimulation of HUVEC monolayers with IL-1β strongly increased the transmigration of THP-1 cells (Fig. 1) as previously shown (22, 23, 24). p-Cresol at 50 μg ml⁻¹ (maximal uremic concentration) and 25 μg ml⁻¹ inhibited IL-1β-induced monocyte transmigration by 45% (P < 0.01 versus IL-1β) and 32% (P < 0.05 versus IL-1β), respectively (Fig. 1A). p-Cresol at the mean uremic concentration (10 μg ml⁻¹) and at normal concentration (1 μg ml⁻¹) had no effect on monocyte transmigration (Fig. 1A). These experiments were also performed in the presence of 4 g dl⁻¹ of HSA since p-cresol is protein bound. In the presence of HSA, p-cresol at 50 μg ml⁻¹ inhibited monocyte transmigration by 55% (P < 0.01 versus IL-1β) (Fig. 1B). The effects of p-cresol observed at 25, 10 and 1 μg ml⁻¹ were not significant (Fig. 1B).

The transmigration assay was also performed with fresh PBMCs of healthy donors, in the presence of p-cresol at 1 and 50 μg ml⁻¹ (Fig. 2). p-Cresol at 50 μg ml⁻¹ inhibited transendothelial migration of PBMCs by 32% (P < 0.05 versus IL-1β). Thus, with or without HSA, p-cresol significantly inhibited THP-1 and PBMC transendothelial migration at concentrations similar to those found in uremic patients.

**p-Cresol inhibited cytokine induction of fractalkine mRNA**

Since p-cresol decreased monocyte transmigration, we investigated its effect on chemokine production by HUVECs by studying MCP-1, IL-8 and fractalkine mRNA levels. Stimulation of HUVECs with IL-1β strongly induced mRNA expression of MCP-1, IL-8 and fractalkine (Table 1) as previously shown (16, 26, 27). p-Cresol did not significantly affect mRNA increase in MCP-1 and IL-8 cytokines (Table 1). In contrast, the increase in fractalkine mRNA expression induced by IL-1β was inhibited by p-cresol by 55% (P < 0.05 versus IL-1β), as shown in Table 1.

**p-Cresol decreased membrane expression of fractalkine on endothelial cells**

In view of the effect of p-cresol on fractalkine mRNA induction, we studied its effects on membrane expression of fractalkine,
which acts as an adhesion molecule for leukocytes. Resting endothelial cells expressed almost no fractalkine, in agreement with published data (16, 18), and IL-1β induced fractalkine expression on HUVECs (Fig. 3). p-Cresol at 50 and 25 μg ml⁻¹ reduced IL-1β-induced fractalkine expression by 46% (\(P < 0.05\) versus IL-1β and \(P < 0.01\) versus IL-1β, respectively) (Fig. 3). The inhibition of fractalkine expression observed with p-cresol at 1 and 10 μg ml⁻¹ did not reach significance (Fig. 3).

p-Cresol decreased soluble form of fractalkine

We also studied the effect of p-cresol on the soluble form of endothelial fractalkine, which acts as a chemokine. Soluble fractalkine was not detectable in resting HUVEC supernatants, whereas IL-1β stimulation strongly induced soluble fractalkine production (Fig. 4). p-Cresol significantly decreased soluble fractalkine production by IL-1β-stimulated HUVECs. A 40% decrease in soluble fractalkine levels was observed with p-cresol at 50 μg ml⁻¹ (\(P < 0.01\) versus IL-1β) (Fig. 4). The decrease in soluble fractalkine production with p-cresol at 25, 10 and 1 μg ml⁻¹ did not reach significance (Fig. 4).

p-Cresol decreased membrane expression of JAM-A

Since JAM-A play an active role in transmigration of leukocytes across endothelial junctions, we have investigated the effect of p-cresol on its membrane expression. Stimulation of HUVECs by IL-1β did not modify JAM-A expression significantly (Fig. 5). p-Cresol inhibited JAM-A expression on stimulated HUVECs by 22% (\(P < 0.05\) versus IL-1β) and by 18% (\(P < 0.05\) versus IL-1β) at 50 and 25 μg ml⁻¹, respectively (Fig. 5). The concentration of 10 and 1 μg ml⁻¹ did not affect JAM-A expression significantly (Fig. 5).

**Discussion**

Our *in vitro* data showed that p-cresol, at uremic concentrations, inhibits transendothelial migration of leukocytes and alters the expression of the membrane-bound and soluble forms of the chemokine fractalkine. In addition, p-cresol decreased membrane expression of the endothelial JAM-A.

Firm adhesion of leukocytes is required before their diapedesis. We have previously shown that p-cresol inhibits leukocyte adhesion to stimulated endothelial cells and cytokine-induced expression of endothelial adhesion molecules intercellular

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<th>Table 1. Effect of p-cresol on mRNA level of fractalkine, IL-8 and MCP-1 in IL-1β-stimulated HUVECs</th>
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<tr>
<td>Control</td>
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<td>Fractalkine mRNA</td>
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<td>MCP-1 mRNA</td>
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<td>IL-8 mRNA</td>
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HUVECs were incubated for 12 h in control medium or IL-1β (50 U ml⁻¹) in the presence or in the absence of p-cresol at maximal uremic concentration (50 μg ml⁻¹) and mRNA levels were studied by RT and comparative PCR. Results are expressed in fold of mRNA induction, as mean ± SEM of three independent experiments. *\(P < 0.05\) versus IL-1β.
Here, we found that p-cresol also decreases membrane expression of fractalkine. After incubation, levels of soluble fractalkine were measured by ELISA in HUVEC supernatants. Data are expressed as mean ± SEM of seven independent experiments. **P < 0.01 versus IL-1β.

**Fig. 4.** Effect of p-cresol on levels of soluble fractalkine in HUVEC supernatants. HUVECs were incubated for 24 h in medium containing IL-1β (50 U ml⁻¹) in the presence of different concentrations of p-cresol. After incubation, levels of soluble fractalkine were measured by ELISA in HUVEC supernatants. Data are expressed as mean ± SEM of seven independent experiments. **P < 0.01 versus IL-1β.

The inhibition by p-cresol of fractalkine expression in cytokine-stimulated endothelial cells occurred at a transcriptional level. However, p-cresol did not affect IL-1β-induced mRNA expression of MCP-1 and IL-8. In endothelial cells, the expression of genes encoding for fractalkine, MCP-1 and IL-8, and also for VCAM-1 and ICAM-1, is regulated by the nuclear factor κB (NF-κB) pathway (36–38). However, this pathway is not sufficient, and other transcription factors must assemble with NF-κB to generate unique transcriptional activating complexes (25, 39). It seems possible that p-cresol acts on transcriptional factors involved in fractalkine, VCAM-1 and ICAM-1 induction but not on those involved in IL-8 and MCP-1 induction.

Previous studies demonstrated that numerous interactions occur between leukocyte adhesion molecules and endothelial junctional molecules to help the transmigrating leukocytes to cross the endothelial junctions (13). The expression of JAM-A, which is involved in these interactions, is decreased by p-cresol. This effect could be a part of the molecular mechanisms by which p-cresol inhibits leukocyte transendothelial migration.
Leukocyte–endothelium interactions are key events for immune functions and for efficient protection against infectious organisms. An association between plasma concentrations of p-cresol and hospitalization for infectious diseases was demonstrated for CRF patients (11). In addition, uremic plasma was shown to inhibit monocyte and granulocyte adhesion to endothelial cells (40). Thus, our in vitro experiments suggested that the uremic retention solute p-cresol may be involved in the immunodeficiency exhibited by CRF patients. p-Cresol is an end product of amino acid catabolism (41), which is >90% protein bound (42), and therefore badly removed by conventional hemodialysis therapies (43). Although static transmigration assay does not accurately reflect leukocyte–endothelium interactions as flow conditions, the in vitro effects of p-cresol were obtained in conditions mimicking uremic plasma concentrations of both p-cresol and albumin, suggesting that they could be of clinical relevance. It must be noticed that a recent work showed that p-cresol is mainly present in the plasma in a conjugated form, the p-cresylsulfate (44). However, clinical adverse effects have been reported to be correlated with high concentration of free p-cresol (11, 45).

In conclusion, our data indicate that the uremic solute p-cresol impairs leukocyte transendothelial migration by altering endothelial adhesive and chemotactic properties. This effect is consistent with the clinical study showing a link between high concentrations of plasma-free p-cresol and hospitalization rates for infectious diseases (11). These findings highlight the importance of developing new therapeutic strategies to improve p-cresol elimination in uremic patients.

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Abbreviations

CRF chronic renal failure
EGM-2 endothelial growth medium 2
FBS fetal bovine serum
HPRT hypoxanthine phosphoribosyl transferase
HSA human serum albumin
HUVEC human umbilical vein endothelial cell
ICAM-1 intercellular adhesion molecule 1
JAM-A junctional adhesion molecule A
MCP-1 monocyte chemotactic protein 1
NF-kB nuclear factor xB
p-Cresol para-cresol
RT reverse transcription
VCAM-1 vascular cell adhesion molecule 1

References


