P-cresol, a uremic retention solute, alters the endothelial barrier function in vitro

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Summary
Patients with chronic renal failure (CRF) exhibit endothelial dysfunction, which may involve uremic retention solutes that accumulate in blood and tissues. In this study, we investigated the in vitro effect of the uremic retention solute p-cresol on the barrier function of endothelial cells (HUVEC). P-cresol was tested at concentrations found in CRF patients, and since p-cresol is protein-bound, experiments were performed with and without physiological concentration of human albumin (4 g/dl). With albumin, we showed that p-cresol caused a strong increase in endothelial permeability after a 24-hour exposure. Concomitant with this increase in endothelial permeability, p-cresol induced a reorganization of the actin cytoskeleton and an alteration of adherens junctions. These molecular events were demonstrated by the decreased staining of cortical actin, associated with the formation of stress fibers across the cell, and by the decreased staining of junctional VE-cadherin. This decrease in junctional VE-cadherin staining was not associated with a reduction of membrane expression. Without albumin, the effects of p-cresol were more pronounced. The specific Rho kinase inhibitor, Y-27632, inhibited the effects of p-cresol, indicating that p-cresol mediates the increase in endothelial permeability in a Rho kinase-dependent way. In conclusion, these results show that p-cresol causes a severe dysfunction of endothelial barrier function in vitro and suggest this uremic retention solute may participate in the endothelium dysfunction observed in CRF patients.

Keywords
P-cresol, endothelial permeability, VE-cadherin, actin, Rho kinase

Introduction
Endothelial dysfunction is a prominent feature in chronic renal failure (CRF) patients (1). Dysfunction is revealed by high serum levels of endothelial activation markers such as endothelial adhesion molecules (2), von Willebrand factor antigen, tissue factor (3) and thrombomodulin (3, 4). Endothelial dysfunction has also been established by abnormal endothelium-dependent vasodilatation response and impaired NO production (5-8). More recently, Harper and collaborators showed that uremic plasma induces a breakdown of the endothelial barrier function for macromolecules in vivo (9). Despite the fact that endothelial dysfunction is clearly demonstrated in CRF patients, little is known about factors and pathophysiological mechanisms responsible for this endothelial dysfunction.
During CRF and partly as a result of impaired renal function, numerous solutes called uremic retention solutes, accumulate progressively in blood and tissues of patients (10, 11). In vitro studies suggest that some of them may participate in the endothelial damage observed in CRF patients: homocysteine induces endothelial apoptosis (12). Advanced Glycation End Products (AGEs) increase endothelial permeability, expression of endothelial adhesion molecules and transmigration of leukocytes through endothelial cells (13, 14), and oxalate decreases endothelial proliferation (15).

P-cresol or 1-4 methyl phenol, is a uremic solute which results from tyrosine and phenylalanine degradation by intestinal bacteria. In the serum of CRF patients, the p-cresol concentration is 10-fold higher than in the general population (16). High level of p-cresol in CRF results from an overgrowth of intestinal bacteria responsible for p-cresol overproduction (17), and also from a reduced clearance. P-cresol is a highly protein-bound compound since in the serum of CRF patients, 90% of total p-cresol was found associated with proteins. Some effects of p-cresol have been characterized in different cell types (18), such as the inhibition of metabolic processes involved in the destruction of bacteria by activated phagocytes (19). Moreover, we have recently shown that p-cresol decreases endothelial proliferation and migration (20). The harmful effects of p-cresol reported in these in vitro works could be supported by a clinical study showing an association between high concentration of free p-cresol and the rate of hospitalization for infectious diseases in CRF patients (21).

One of the most important functions of endothelial cells is to constitute a barrier between blood and neighbouring tissues, the barrier which actively regulates the exchange of plasma proteins and circulating cells through endothelial cell-to-cell junctions (22). The endothelial barrier function is dependent on the actin cytoskeleton organization (23). It has been demonstrated that the small GTPase Rho protein and its target Rho kinase (ROCK) play a central role in the regulation of endothelial permeability by controlling cell shape via the actin cytoskeleton (23). Upon activation, Rho can associate with Rho kinase which in turn leads to an increase in myosin light chain (MLC) phosphorylation. This increased MLC phosphorylation leads to stress fiber formation which is responsible to cell contraction and gap appearance between adjacent cells (23, 24). In addition to the actin cytoskeleton role, the barrier function involves specialized protein complexes localized at cell-to-cell contacts that form the cellular junctions (25). Among these, adherens junctions are constituted by a transmembrane vascular specific cadherin, VE-cadherin, which mediates homophilic interactions between adjacent cells through its extracellular domain. In the cells, VE-cadherin is linked to the actin cytoskeleton via the interactions of its cytoplasmic tail with catenins (25, 26).

The observation that the endothelial barrier function is impaired by uremic plasma in frogs (9) suggests that uremic solutes play a role in this dysfunction. Hence, we investigated whether the uremic solute p-cresol may be involved in loss of endothelial barrier function in vitro by measuring endothelial permeability. Since the endothelial barrier function depends largely on the integrity of the protein complexes that form cell-to-cell contacts, we investigated the in vitro impact of p-cresol on VE-cadherin localization and on actin cytoskeleton organization of endothelial cells.

**Methods**

**Reagents and antibodies**

P-cresol was obtained from Supelco (Sigma-Aldrich, Saint Quentin Fallavier, France). Bovine serum albumin, sodium azide and 70 kDa fluorescein-conjugated Dextran (FITC-dextran) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and methanol from Carlo Erba (Milano, Italy). Monoclonal antibody against VE-cadherin (clone 1G11) and irrelevant control IgG were purchased from Coulter Immunotech (Marseille, France). FITC-conjugated sheep anti-mouse Ig F(ab’)2, fraction (Silenus) was from Eurobio (Les Ulis, France). Texas-red-phalloidin was obtained from Molecular Probes (New York, NY). QIFIKIT® beads were from Dako (Trappes, France). TFN α was purchased from Tebu (Le Perray en Yvelines, France). The Cytotoxicity Detection kit was from Roche (Grenoble, France). Y-27632 was from Calbiochem (USA).

EGM-2 medium (supplemented with 2% fetal bovine serum) was purchased from Clonetics Biowhitthaker (Verviers, Belgium) and human albumin solution 20% was purchased from LFB (Courtaboeuf, France). Phosphate buffer saline (PBS), RPMI 1640 medium with glutamax-1, trypsin-EDTA solution, gelatin, BSA fraction V, and penicillin-streptomycin were obtained from Invitrogen (Cergy-Pontoise, France). Fetal bovine serum was from Dominique Dutscher (Bramath, France). Cell culture inserts were obtained from BD Biosciences (Meylan, France).

**Endothelial cell culture and treatment with p-cresol**

Human umbilical vein endothelial cells (HUVEC) were recovered from umbilical cord vein by collagenase digestion according to Jaffe, et al (27). Cells were seeded on gelatin-coated culture flasks 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 on the different supports used in this work until they reached confluence. Then, medium was removed and cells were washed briefly with RPMI supplemented with 0.5% BSA. After this wash, HUVEC were incubated for 24 hours in EGM-2 medium containing different concentrations of p-cresol or 0.5% methanol (control medium).
As p-cresol is protein-bound and since endothelial culture medium was poor in protein concentration (2% serum), the EGM-2 medium was supplemented with 4% of human albumin (EGM-2-HSA) to mimic the in vivo human serum albumin concentration (40 g/l) and to increase the protein-bound fraction of p-cresol. Experiments were also performed in EGM-2 medium not supplemented with 4% human albumin.

In experiments performed with Rho kinase inhibitor, Y-27632 was added at 10 µg/ml simultaneously with p-cresol during 24 hours.

**Endothelial cell viability**

Cell viability in presence of p-cresol was monitored by trypan blue exclusion. Cells were incubated with medium alone, control medium (containing 0.5% methanol), or medium containing 10 µg/ml or 50 µg/ml of p-cresol with or without 4% human albumin. After 24 hours, cells were then detached with a 0.05% trypsin-0.02% EDTA solution and washed with PBS-20% fetal bovine serum. Trypan blue was added and the percentage of cells excluding trypan blue was counted.

Lactate dehydrogenase (LDH) release in culture medium was measured according to the manufacturer’s instructions. LDH release by HUVEC was measured after 4 hours and 24 hours of incubation with medium alone, control medium, or medium containing 10 µg/ml or 50 µg/ml of p-cresol. Then, 100 µl of medium was taken to measure LDH activity by a two-step reaction that produces formazan, whose appearance is monitored at 490 nm on an ELISA reader (Dynex technologies, France). LDH release was not investigated in medium containing 4% albumin because high concentrations of proteins interfere with this test.

**Measurement of HUVEC monolayer permeability**

Cells were seeded at 60,000 cells per wells on fibronectin-coated cell culture inserts (porosity: 1 µm; diameter: 6 mm) and allowed to grow to confluence. Following HUVEC exposure to medium containing p-cresol or control medium, the permeability test was performed in the following way: cells were rapidly washed with HBSS supplemented with 0.5% BSA. Then, 600 µl of HBSS-0.5% BSA was added to the lower compartment, and 100 µl of HBSS-0.5% BSA containing FITC-dextran at 50 µM was added to the upper compartment. The cells were incubated at 37°C in 5% CO₂ for 90 minutes, and then an aliquot of 100 µl was taken from the lower compartment to establish the FITC-dextran clearance. Fluorescence was determined by the Cytofluor® Series 4000 Fluorescence Multi-well Plate Reader (PerSeptive Biosystems, Framingham, MA). The excitation wavelength was at 485 nm and the emission wavelength at 530 nm. A serial dilution of the FITC-dextran was made to obtain the linear relationship between fluorescence intensity (arbitrary units) and the corresponding concentration of FITC-dextran. The fluorescence intensity obtained for each sample was converted into picomole of FITC-dextran. The permeability coefficients were expressed as picomoles of FITC-dextran per minute.

**Nuclear staining**

HUVEC were cultured on fibronectin-coated glass coverslips and allowed to grow to confluence. After HUVEC exposure to medium containing p-cresol or control medium for 24 hours, cells were briefly washed with PBS preheated at 37°C, and then fixed with 3% paraformaldehyde in PBS for 30 minutes. Cells were permeabilized with 0.2% triton X-100 in PBS for 5 minutes. After two washes in PBS, samples were incubated for nuclear staining with diamidinophenylindol (DAPI) at 1 mg/ml in DAPI Buffer (100 mM NaCl, 10 mM Tris pH 7.4 and 10 mM EDTA) for 30 min at 37°C. Nuclear count was performed under videomicroscopy coupled with a software system allowing image analysis (Biocom, Les Ulis, France).

**Confocal microscopy studies of actin filament and VE-cadherin distributions**

All washes and incubations were carried out at room temperature. HUVEC were cultured on fibronectin-coated glass coverslips and allowed to grow to confluence. After HUVEC exposure to medium containing p-cresol or control medium for 24 hours, cells were briefly washed with PBS preheated at 37°C and then fixed with 3% paraformaldehyde in PBS for 30 minutes. Cells were permeabilized with 0.2% triton X-100 in PBS for 5 minutes. Saturation was done with 5% fetal bovine serum in PBS, for 10 minutes. For actin staining, rhodamine-labeled phalloidin (texas-red-phalloidin) diluted in PBS-3% fetal bovine serum was applied to the coverslips for one hour. For VE-cadherin staining, coverslips were incubated with primary monoclonal antibody (mAb) against VE-cadherin or with an irrelevant control antibody (IgG1 isotypic control) in PBS-3% fetal bovine serum for one hour, washed three times with PBS and incubated one hour with FITC-conjugated secondary antibody in PBS-3% fetal bovine serum at room temperature. After three rinses in PBS, coverslips were mounted onto slides using mowiol. For the double staining (actin and VE-cadherin), texas-red-phalloidin was added with the FITC-conjugated secondary antibody. Slides were evaluated by confocal laser microscopy.

**Flow cytometry analysis of VE-cadherin expression**

Confluent HUVEC monolayers incubated with p-cresol or control medium were detached with a pre-warmed 0.05% trypsin-0.02% EDTA solution for 30 seconds at 37°C. Cells were washed with PBS-0.1% bovine serum albumin-0.1% sodium azide (PBS-BSA-AZ) and incubated with 50 µl of mAb against VE-cadherin diluted at 20 µg/ml or with irrelevant control mAb at 10 µg/ml for one hour at 4°C. After being washed with PBS-
Figure 1: Effect of p-cresol on endothelial permeability. HUVEC were treated with control medium, with p-cresol at different concentrations or with TNF α during 24 hours in presence (A) or in absence of albumin (B). Then, permeability of the monolayers was quantified. P-cresol induced a significant increase in endothelial permeability at 10 and 50 µg/ml. Data are expressed as mean ± SEM of 6 independent experiments performed in triplicate. *p<0.05 versus control, **p<0.01 versus control, ***p<0.001 versus control. Nuclear count (C) of nuclei stained with DAPI (D) was performed without albumin. Data are expressed as mean ± SEM of 3 independent experiments. (Bar, 50 µm).
BSA-AZ, cells were labeled with 100 μl of FITC-conjugated secondary antibody for 45 minutes at 4°C. After two additional washes, endothelial cells were analyzed using an Epics® XL flow cytometer (Beckman-Coulter, Roissy, France). Mean fluorescence intensity on the whole cell population was calculated by the System II™ software (Beckman-Coulter, Roissy, France) and was expressed in arbitrary units.

Quantification was done with the QIFI assay (Quantitative Immunofluorescent Indirect Assay), which serves to obtain a linear relationship between mean fluorescence intensity (arbitrary units) and number of binding sites of mAb per cell (28).

Briefly, the fluorescence intensity measured on the flow cytometer was converted into mAb binding sites per cell by standard beads (QIFIKIT®), labeled with the FITC-conjugated secondary antibody. A linear regression curve was drawn by plotting the mean fluorescence intensity measured on each bead sample and the known number of mAb binding sites per cell for each bead. The mean fluorescence intensity measured on cells labeled with the mAb against VE-cadherin and corrected with isotype control value was converted with the standard curve equation into the corresponding number of mAb binding sites per cell. Endothelial cells were considered positive when they expressed more than 5000 mAb binding sites per cell.

Statistical analysis
Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with the Prism® software (GraphPad Software Inc, San Diego, CA). Differences were assessed by ANOVA followed by a Turkey’s multiple comparison test. A p value lower than 0.05 was considered significant.

Results
P-cresol did not affect cell viability
HUVEC were incubated with medium alone, control medium containing 0.5% methanol, and medium containing p-cresol at 10 μg/ml and at 50 μg/ml during 24 hours with or without human albumin. Whatever the conditions, the cell viability monitored by trypan blue exclusion was always > 90%.

LDH release was also investigated after 4 hours and 24 hours of incubation with p-cresol to determine if it could cause membrane damage. The LDH release in presence of p-cresol was similar to that observed with control medium or with medium alone (data not shown).

Thus, p-cresol did not alter cell viability and membrane integrity in the range of concentrations used.

P-cresol increased endothelial permeability
To determine whether p-cresol modifies the endothelial barrier function in vitro, confluent endothelial cells cultured on filters were exposed to p-cresol at different concentrations in the presence of human albumin during 24 hours. The concentrations used were 1 μg/ml (concentration found in healthy subjects), 5, 10 μg/ml (concentrations found in CRF patients; 10 μg/ml being the mean concentration) and 50 μg/ml. After 24 hours of incubation, the permeability of the endothelial monolayer was measured (Fig. 1A). We showed that p-cresol caused a significant increase in endothelial monolayer permeability at 10 and 50 μg/ml; the permeability was increased by 85% relative to control (p<0.05 versus control) and by 114% relative to control (p<0.01 versus control) respectively. TNFα, a cytokine known to strongly increase the endothelial permeability (29), was used as a positive control. The permeability obtained with p-cresol at 50 μg/ml was not significantly different from that obtained with TNFα at 500 U/ml (Fig. 1A). Thus, p-cresol triggered an increase in endothelial permeability comparable to the one induced by TNFα.

This functional test was also performed in a medium not supplemented with human albumin in order to determine whether an increase in the free fraction of p-cresol could be more deleterious (Fig. 1B). In this condition, the effects of p-cresol on endothelial permeability were more marked: at 10 μg/ml of p-cresol, the permeability was increased by 127% relative to control (p<0.01 versus control), and at 50 μg/ml of p-cresol, the permeability was increased by 153% relative to control (p<0.001 versus control). The increase in permeability observed at 1 and 5 μg/ml was not significantly different from that obtained with control medium.

The effect of p-cresol on endothelial permeability was also investigated after 5 and 10 hours of incubation with the highest concentration of p-cresol (50 μg/ml) in the presence or in the absence of albumin. We did not detect a significant increase in endothelial permeability after 5 and 10 hours of incubation (data not shown).

We have previously shown that p-cresol decreases the proliferation of sub-confluent endothelial cells (20). Therefore, we analyzed the effect of p-cresol on the density of confluent endothelial cells, to determine if the increase in endothelial permeability induced by p-cresol could be attributed to a reduction of cell density. Without albumin (Fig. 1C, 1D), p-cresol at 10 μg/ml did not decrease cell density. At 50 μg/ml of p-cresol, the decrease in cell density was 23%. The results obtained with albumin were similar (data not shown). Therefore in confluent cells, the increase in endothelial permeability induced by p-cresol at 10 μg/ml could not be attributed to a reduction of cell density.

Effect of p-cresol on the cell shape and the actin cytoskeleton organization
Since the actin cytoskeleton organization largely influences endothelial monolayer permeability, we investigated whether p-cresol triggers alterations of the actin filament network. Confluent HUVEC were incubated during 24 hours in the presence of p-cresol at 10 or 50 μg/ml, with (Fig. 2A-2C) or without (Fig. 2D-2F) albumin.
Figure 2: P-cresol induced the formation of stress fibers. HUVEC were treated with control medium (A), with p-cresol at 10 µg/ml (B) or 50 µg/ml (C) during 24 hours in presence of 4% human albumin. Panels D, E and F illustrate the same experiments performed without albumin. The actin cytoskeleton was revealed by Texas-red phalloidin staining. This figure is representative of 4 independent experiments. (Bar, 20 µm).

Figure 3: P-cresol induced a decrease in the VE-cadherin staining at cell-to-cell junctions. HUVEC were treated with control medium (A), with p-cresol at 10 µg/ml (B) or 50 µg/ml (C) during 24 hours in presence of 4% human albumin. Panels D, E and F illustrate the same experiments performed without albumin. Cells were stained for VE-cadherin by indirect immunofluorescent staining. Arrows indicate the presence of gaps. This figure is representative of 4 independent experiments. (Bar, 20 µm).
In the control HUVEC, fluorescence staining revealed F-actin was concentrated along the cell border and in fine F-actin filaments across the cells (Fig. 2A, 2D) as already described (30). These cells were well spread and displayed a typical cobblestone morphology of an endothelial monolayer. In contrast, when cells were exposed to p-cresol at 10 µg/ml, the dense peripheral band of junctional F-actin was strongly decreased (Fig. 2B, 2E). Moreover, stress fibers appeared across these cells and some of the cells adopted an elongated shape. The effect was more pronounced at the highest concentration of p-cresol (50 µg/ml). Actin stress fibers were more numerous and longer; nearly all the cells were highly elongated (Fig. 2C, 2F).

Cells treated with p-cresol at 50 µg/ml exhibited a morphology more elongated in absence of albumin than in presence of albumin.

**Alterations of VE-cadherin distribution caused by p-cresol**

As alteration of cell shape and disorganization of the cellular junctions could provide a structural basis for the impairment of barrier properties of endothelial cells, we investigated the effects of p-cresol on the localization of the VE-cadherin. Confluent HUVEC were treated with p-cresol at 10 µg/ml and 50 µg/ml during 24 hours in presence (Fig. 3A-3C) or in absence of albumin (Fig. 3D-3F).

In the control HUVEC, VE-cadherin was restricted to the cell-to-cell junctions and exhibited a pattern characteristic of well-established contacts with continuous staining along the cell border (Fig. 3A, 3D), as already described (31). VE-cadherin staining was specific since HUVEC were not stained with an irrelevant control mAb (data not shown). With albumin, treatment with p-cresol at 10 µg/ml altered the junctional staining of VE-cadherin. At 10 µg/ml of p-cresol, labeling intensity decreased along the cell border (Fig. 3B). When HUVEC were exposed to p-cresol at 50 µg/ml, the intensity of VE-cadherin staining was more reduced at cell-to-cell junctions compared to cells exposed to p-cresol at 10 µg/ml (Fig. 3C).

Without albumin, the effects of p-cresol were more pronounced. The decrease in the junctional staining of VE-cadherin was more important than in presence of albumin with the appearance of punctate staining without continuity (Fig. 3E, 3F). Furthermore, with p-cresol at 10 and 50 µg/ml, gaps between adjacent endothelial cells could be seen where junctional protein staining was lost (Fig. 3E, 3F).

**Membrane expression of VE-cadherin was not affected during p-cresol exposure**

To determine whether VE-cadherin undergoes degradation or internalization during p-cresol exposure, membrane expression of this protein was quantified by flow cytometry after a 24-hour incubation with p-cresol. Irrespective of the dose of p-cresol used and the presence of albumin, the membrane expression of VE-cadherin on the cell surface remained unchanged (Fig. 4), indicating that the junctional decrease of VE-cadherin staining was not attributable to a degradation or internalization of this protein.

**Colocalization between VE-cadherin and actin was prevented by p-cresol**

VE-cadherin is involved in the connections between the endothelial cell membrane and its underlying actin cytoskeleton. The effect of p-cresol, on these protein-protein interactions was investigated and visualized by a double staining for VE-cadherin (in green) and actin cytoskeleton (in red).

As shown in Figure 5A and 5C, the double staining of HUVEC incubated in control medium displays yellow labeling indicating the colocalization of actin and VE-cadherin. In contrast, in presence of p-cresol, the remaining junctional VE-cad-
Rho kinase is involved in the increase in endothelial permeability induced by p-cresol

To determine if the Rho/Rho kinase pathway could be involved in the increase in endothelial permeability, HUVEC were simultaneously treated with p-cresol and Y-27632, a specific Rho kinase inhibitor (32) during 24 hours. These experiments were performed without albumin since the effects induced by p-cresol without albumin were higher than with albumin. Y-27632 inhibited by 71% the increase in endothelial permeability induced by p-cresol (Fig. 6A). The permeability values obtained in the presence of p-cresol and Y-27632 was similar to those obtained with the control medium. Y-27632 attenuated the formation of stress fibers, and actin staining was conserved along the cell borders to the same extent than in control cells (Fig. 6B). The thinner actin staining observed in cells treated with Y-27632 (Fig. 6B) is due to a beginning of actin depolymerization induced by the inactivation of the Rho/Rho kinase pathway as previously described (33). As a consequence of the prevention of stress fiber formation, the cell shape was not altered and seemed close to the control cells. Furthermore, the number of

Figure 5: Colocalization between VE-cadherin and actin was prevented by p-cresol. HUVEC were treated with control medium (A), with p-cresol at 10 µg/ml (B) or 50 µg/ml (C) during 24 hours in presence of 4% human albumin. Panels C and D illustrate the same experiments performed without albumin. Cells were double stained for actin cytoskeleton with texas-red-phalloidin in red and for VE-cadherin by indirect immunofluorescent staining in green. This figure is representative of 4 independent experiments. (Bar, 20 µm).

Figure 6: Rho kinase is involved in the increase in endothelial permeability induced by p-cresol. HUVEC were treated with control medium or p-cresol at 50 µg/ml in the presence of Y-27632 without albumin (A, B and C). Permeability of the monolayers and quantified (A) Data are expressed as mean ± SEM of 4 independent experiments performed in triplicate. **p<0.01 versus control. B: cells were stained for actin cytoskeleton with texas-red-phalloidin. C: cells were stained for VE-cadherin. Figures B and C are representative of 5 independent experiments. (Bar, 20 µm).
gaps between cells is strongly decreased. The VE-cadherin staining was also preserved at the cell junctions to the same extent than in control cells (Fig. 6C).

**Discussion**

In the present work, we have studied the effect of the uremic retention solute p-cresol on the barrier function of endothelial cells *in vitro*. We showed that p-cresol strongly increased the endothelial monolayer permeability. This increase in endothelial permeability was associated with an alteration of adherens junctions and with an alteration of actin cytoskeleton organization. P-cresol induced a decrease in VE-cadherin and cortical actin staining at the intercellular junctions, in parallel with a rise in stress fibers across the cells.

In quiescent cells, actin is predominantly found in a dense peripheral band at the cell borders and reinforce the adherens junctions. The small GTPase Rho protein and its target ROCK actively regulate the actin cytoskeleton organization (23). Upon activation of Rho, ROCK phosphorylates and inactivates a MLC phosphatase (PP1). This decrease in phosphatase activity is responsible for an increase in MLC phosphorylation leading to the formation of stress fibers and the opening of cell junctions (23, 24). In our study, we have demonstrated that the increase in endothelial permeability induced by p-cresol was partly mediated by the Rho/Rho kinase pathway since the specific ROCK inhibitor Y-27632 inhibits the formation of stress fibers and the increase in endothelial permeability. We cannot exclude that other partners contribute to the remodelling of the actin cytoskeleton. In addition, how p-cresol could activate ROCK remains to be determined.

Adherens junctions constituted by VE-cadherin which mediated homotypic and homophilic interactions, are also essential to maintain the barrier function of endothelial cells. However, these interactions are not sufficient to achieve barrier function. Indeed, the linkage between actin and VE-cadherin via cytoplasmic protein named catenins come to strengthen the intercellular adhesion mediated by VE-cadherin (26). Hence, the integrity of this linkage between junctional proteins and the cytoskeleton is crucial for endothelial cells to exert barrier function *in vitro* and *in vivo* (34–36). We have demonstrated that p-cresol induces a decrease in the amount of VE cadherin at the cell junctions. Furthermore, the remaining junctional VE-cadherin seems to be dissociated from the actin cytoskeleton, as suggested by the delocalization of the actin cytoskeleton and the VE-cadherin staining. This dissolution of adherens junctions participates to the endothelial barrier dysfunction induced by p-cresol since Y-27632 allows the preservation of VE-cadherin at the cell junctions and prevents the increase in endothelial permeability.

The decrease in the VE-cadherin staining along cell-to-cell junctions could be attributed to a decrease in membrane expression of VE-cadherin, to the elongation of cell shape induced by p-cresol or to a redistribution of VE-cadherin to the whole cell surface. We demonstrated that membrane expression of VE-cadherin in HUVEC was not modified by p-cresol. Therefore, the decrease in junctional staining of VE-cadherin from intercellular contacts was not attributable to internalization or shedding processes but rather to a change in cell shape and/or a diffuse redistribution of VE-cadherin to the cell surface. The redistribution of VE-cadherin to the whole cell surface has been already described in HUVEC exposed to an anti VE-cadherin monoclonal antibody (35).

We have previously shown that p-cresol decreases endothelial proliferation of sub-confluent cells (20). Therefore, we performed the permeability experiments with very confluent cells in order to avoid an effect of p-cresol on endothelial cell density. In these conditions, p-cresol at 10 μg/ml induced a less than 5% decrease in cell density, and at 50 μg/ml p-cresol induced a 23% decrease. These decreases in cell density are not sufficient to explain the strong increase in endothelial permeability induced by p-cresol. In addition, the effects observed with p-cresol did not result from an unspecific toxic effect since the endothelial cell viability and membrane integrity were not modified by p-cresol.

The effects were observed at p-cresol concentration usually found in the serum of CRF patients and in presence of physiological concentration of albumin. In CRF patients, total p-cresol concentration is 10-fold higher than in healthy subjects (16). Since 90% of total p-cresol is protein-bound in CRF patients and 100% in healthy subjects, the free p-cresol concentration is also increased compared to the general population (16). Recently, a clinical study demonstrated that hypoalbuminemia, which is usually found with CRF (37), augmented the increase in free p-cresol concentration in these patients (21). In addition, the authors showed an association between free plasma p-cresol levels and hospitalization because of infection (21). Because in CRF patients the free p-cresol concentration is increased, we performed also experiments in absence of albumin to increase the free fraction of p-cresol. The results obtained with p-cresol in this condition were more pronounced especially at 50 μg/ml. These differences suggest that the free p-cresol fraction is the more active fraction, but these results do not exclude that the protein-bound fraction may have an effect.

The mechanism of p-cresol action on endothelial cells is not yet known. Previous work has demonstrated that p-cresol induced a depletion of the intracellular glutathione levels in rat liver slices (38). One could hypothesize that the mechanism by which p-cresol causes endothelial dysfunction is similar. However, determining the exact molecular mechanisms by which p-cresol initiates these effects in HUVEC require further study.

The present study suggests that p-cresol may be involved in increase in endothelial permeability in CRF patients. The obser-
observation that uremic plasma increases endothelial permeability in frogs (9) supports this hypothesis. In addition, one could think that other uremic toxins, such as AGEs, contribute actively to the in vivo observed increase in endothelial permeability. Impairment of endothelial integrity is critical for atherogenesis (39). Moreover, many risk factors that are causally related to the development of cardiovascular diseases cause an increase in endothelial permeability in vitro or in vivo. These risk factors include high levels of LDL (40), oxidized LDL (41), nicotine (42) and glucose (43). Furthermore, AGEs also increase endothelial permeability (13). Thus, if the effects of p-cresol observed in vitro reflect the in vivo situation, one could think that p-cresol could play a role in cardiovascular diseases in CRF patients.

P-cresol occupies a specific place among uremic retention solutes: it is lipophilic and protein-bound, and removal of such compounds with the current dialytic strategies is at present unsatisfactory (44). P-cresol has been demonstrated to inhibit several biochemical, biological and physiological functions in vitro (18). Our data underscore one more biological effect of the protein-bound uremic solute p-cresol and suggest that this compound may be involved in endothelial dysfunction in CRF patients. In vivo studies are now necessary to demonstrate the implication of p-cresol in cardiovascular diseases.

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References