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The uremic solutes p-cresol and indoxyl sulfate inhibit endothelial proliferation and wound repair

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Background. Cardiovascular diseases are the major causes of mortality in uremic patients, and the vascular endothelium is dysfunctional in uremia. We hypothesized that uremic retention solutes may be among the factors involved in this endothelial dysfunction. We therefore investigated the in vitro effect of a large panel of uremic retention solutes (guanidino compounds, polyamines, oxalate, myoinositol, urea, uric acid, creatinine, indoxyl sulfate, indole-3-acetic acid, p-cresol, hippuric acid, and homocysteine) on endothelial proliferation. In addition, we tested the effect of uremic solutes that altered proliferation on endothelial wound repair.

Methods. Human umbilical vein endothelial cells (HUVEC) were incubated with uremic retention solutes at concentrations in the range found in uremic patients. Protein-bound uremic solutes were also tested in the presence of 4% human albumin. Then, we determined the effect of each uremic solute on endothelial proliferation by a 5-bromo-2-deoxy-uridine (BrdU) labeling assay. In addition, confluent endothelial monolayers were injured, incubated with uremic solutes that altered endothelial proliferation, and the surface of the wound was measured at different intervals by image analysis.

Results. Endothelial proliferation was inhibited by two protein-bound uremic retention solutes: p-cresol and indoxyl-sulfate. Inhibition of endothelial proliferation by p-cresol was dose-dependent. Moreover, p-cresol and indoxyl sulfate decreased endothelial wound repair. The presence of albumin did not affect the inhibitory effect of these solutes on endothelial proliferation, but the decrease in endothelial wound repair was less marked in the presence of albumin.

Conclusion. We demonstrated that both p-cresol and indoxyl sulfate decrease endothelial proliferation and wound repair. These solutes could play a role in endothelial dysfunction observed in uremic patients.

Chronic renal failure (CRF) patients display endothelial dysfunction characterized by an impairment of endothelium-dependent vasodilatation [1], and an increase in plasma levels of endothelial molecules involved in vascular tone, hemostasis, or adhesion [2–5]. It has been suggested that uremic solutes, which accumulate in CRF patients, are among the factors involved in this endothelial dysfunction. In vitro, some uremic retention solutes induce endothelial dysfunction. Homocysteine up-regulates the gene coding for a stress protein [6], p-cresol inhibits endothelial cell response to inflammatory cytokines [7], and advanced glycation end products (AGEs) up-regulate the expression of endothelial adhesion molecules [8], and increase endothelial permeability [9].

Endothelial dysfunction plays an important role in the development of cardiovascular diseases [10], which are the leading cause of mortality in CRF patients [11]. The physical and metabolic integrity of the endothelium is a critical element in the prevention or delay of vascular diseases. In the case of endothelial injury, an active repair process is required. In this view, endothelial ability to proliferate and migrate plays an important role in the healing of endothelial injury [12]. It seemed of interest to analyze the effect of a large panel of uremic solutes on endothelial cells, and especially on endothelial functions involved in repair of endothelial injury. We therefore investigated the in vitro effect of 16 known uremic retention solutes on endothelial proliferation. These solutes belong to two groups with different physicochemical characteristics: the free water-soluble compounds (urea, creatinine, myoinositol, oxalate, uric acid, guanidino compounds, polyamines), and the protein-bound compounds (indoxyl sulfate, indole-3-acetic acid, p-cresol, hippuric acid, and homocysteine). In addition, we studied the effect of uremic solutes that altered proliferation on endothelial repair after wounding in vitro.

Key words: endothelium, uremic retention solutes, proliferation, wound repair, uremia.

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METHODS

Reagents

All uremic solutes, listed in Table 1, were obtained from Sigma (Saint Quentin Fallavier, France), except

Table 1. Individual solutes under evaluation for screening of endothelial viability and proliferation

Uremic compound	Concentration μg/mL	Molecular weight D	References
Free water-soluble			
Urea	1200	60.1	14
Creatinine	60	113.1	15
Myoinositol	100	180.2	16
Oxalate	5	90	17
Uric acid	80	168.1	18
Methylguanidine	0.33	73.1	19
γ-guanidinobutyric acid	0.02	145.2	14
β-guanidinopropionic acid	0.03	131.1	20
Guanidinosuccinic acid	2.5	175.1	20
Spermine	0.01	202.3	21
Spermidine	0.049	145.2	21
Protein-bound			
P-cresol	10	108.1	22
Hippuric acid	50	179.2	18
Indole-3-acetic acid	3.5	175	23
Indoxyl sulfate	25	251.3	24
Homocysteine	2.7	135.2	25

oxalate, which was from Prolabo (Lyon, France). Methanol was purchased from Carlo Erba (Milano, Italy), ammonium hydroxide from Sigma, and Tris from Euromedex (Mundolsheim, France). Endothelial growth medium (EGF)-2 medium was from Clonetics Biowhittaker (Verviers, Belgium). Trypsin-ethylenediaminetetraacetic acid (EDTA) solution, Trypan Blue, RPMI medium and gelatin were obtained from Invitrogen (Cergy-Pontoise, France). Fetal bovine serum (FBS) was from Dominique Dutscher (Brumath, France). Human serum albumin (HSA) solution 20% was purchased from LFB (Courtaboeuf, France). 5-bromo-2′-deoxy-uridine (BrdU) Labeling and Detection Kit III was from Roche (Meylan, France). Annexin V-fluoroscein isothiocyanate (FITC) Kit was from Immunotech (Marseille, France), tumor necrosis factor (TNF) from Tebu (Le Perray en Yvelines, France), and cycloheximide from Sigma.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cord vein by collagenase digestion as previously described [13]. Cells were seeded on gelatin-coated culture plates and grown in EGM-2 medium under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37°C). Cells were then detached with a 0.05% trypsin-0.02% EDTA solution and subcultured to the second passage on gelatin-coated culture plates.

Screening of uremic solutes

The concentrations of the uremic solutes used in this study (Table 1) are in the range of those reported in CRF. Protein-bound uremic solutes were analyzed in absence and in presence of HSA at the concentration found in human serum (4 g/dL). Each test was performed in duplicate. P-cresol was diluted from a stock solution prepared in methanol, homocysteine from a stock solution prepared in Tris buffer 10 mmol/L, and hippuric acid from a stock solution prepared in ammonium hydroxide. The other solutes were diluted from stock solutions prepared in water. Uremic solutes were compared with their respective controls (methanol, Tris, ammonium hydroxide, or water). Solutes were diluted at least 1/500 in culture medium to reach mean uremic concentrations. For dose-response experiments, two solutes, p-cresol and indoxyl sulfate, were tested at concentrations higher than mean uremic concentrations. The maximal concentrations of p-cresol and indoxyl sulfate were obtained by diluting both stock solutions at 1/200.

The effect of diluents (controls) was also tested. The dilutions of methanol, Tris buffer, and ammonium hydroxide were, respectively, 1/200 (dilution corresponding to the highest dose of p-cresol, 50 μg/mL), 1/250,000 (dilution equivalent to that of homocysteine at uremic concentration), and 1/2000 (dilution equivalent to that of hippuric acid at uremic concentration). Since several solutes were diluted in water, the dilution of water tested was the lowest 1/200 (dilution corresponding to the highest dose of indoxyl sulfate, 250 μg/mL).

Endothelial viability assay

The effect of uremic solutes on cell viability was determined by Trypan Blue exclusion. In brief, HUVECs cultured on 24-well culture plates were incubated during 24 hours with uremic solutes or with their respective controls. After 24 hours, endothelial cells were detached with trypsin-EDTA solution, Trypan Blue solution was added, and the percentage of cells excluding Trypan Blue was determined.

Endothelial cell proliferation assay

The effect of uremic retention solutes or controls (diluents of solutes) on endothelial cell proliferation was assessed by BrdU incorporation into cellular DNA. HUVECs at a concentration of 10,000 cells per well were seeded on gelatin-coated 96-well culture plates in EGM-2 medium and cultured during 2 days. Cells were washed with RPMI, and then uremic solutes at mean uremic concentration (Table 1) or controls were added overnight with BrdU. For protein-bound uremic solutes, experiments were undertaken without and with 4 g/dL HSA added to the medium. Cell incorporation of BrdU was measured by enzyme linked immunosorbent assay (ELISA) with the BrdU Labeling and Detection Kit III, according to the manufacturer's instructions. In brief, cells were fixed, cellular DNA was partially digested by nuclease treatment, and incorporated BrdU was detected

with anti-BrdU monoclonal antibodies (mAb) conjugated with peroxidase. The absorbance was measured by a microtiter plate reader (UVmc^2) (Safas, Monaco) at 405 nm and was directly correlated to the level of BrdU incorporation into cellular DNA.

Endothelial apoptosis assay

The effect of p-cresol and indoxyl sulfate on cell apoptosis was determined with the annexin V–FITC kit. HUVECs cultured on 6-well culture plates were incubated with p-cresol at 10 μg/mL, 25 μg/mL, or 50 μg/mL, or with indoxyl sulfate at 25 μg/mL, 50 μg/mL, 125 μg/mL, or 250 μg/mL. Nontreated wells received a dilution of methanol equivalent to p-cresol at the highest concentration or a dilution of water equivalent to indoxyl sulfate at the highest concentration. As a positive control of apoptosis, some cells were incubated with TNF at 25 ng/mL and cycloheximide at 10 μg/mL. After 24 hours, endothelial cells were detached with trypsin-EDTA solution, washed in phosphate-buffered saline (PBS) containing 20% FBS, centrifuged, and washed with ice-cold PBS. After centrifugation, the pellet was resuspended in binding buffer containing annexin V–FITC and propidium iodide, mixed gently, and incubated on ice for 10 minutes in the dark. The samples were then read in an Epics® XL flow cytometer (Beckman-Coulter, Roissy, France) at 488 nm excitation. A 515 nm bandpass filter for FITC detection, and a >600 nm filter for propidium iodide detection were used. The percentage of annexin V–positive cells in the endothelial cell population was determined by the System II™ software (Beckman-Coulter, Roissy, France).

Endothelial wound repair

Confluent endothelial monolayers cultured on gelatin-coated 24-well culture plates were wounded with a yellow tip, washed with RPMI, and p-cresol or indoxyl sulfate at different concentrations was added. To avoid differences due to wound size, experiments were performed on wounds of equivalent sizes. Experiments were undertaken without and with 4 g/dL HSA added to the medium. Cell wound repair was analyzed under videomicroscopy coupled with a software system allowing image analysis (Biocom, Les Ulis, France). The surface of the wound was measured at time 0, and after 5 and 24 hours. Cell wound repair was calculated by subtracting the area obtained in the presence of uremic solutes or controls at 5 and 24 hours of incubation from the area of the original wound (measured at time 0 before the addition of uremic solutes or controls). The area obtained after cell migration was expressed as a percentage of the area of the original wound, considered as 100%. A linear regression curve was obtained by plotting the values of areas obtained after cell migration versus time. The slope of the curve reflects the speed of cell wound repair.

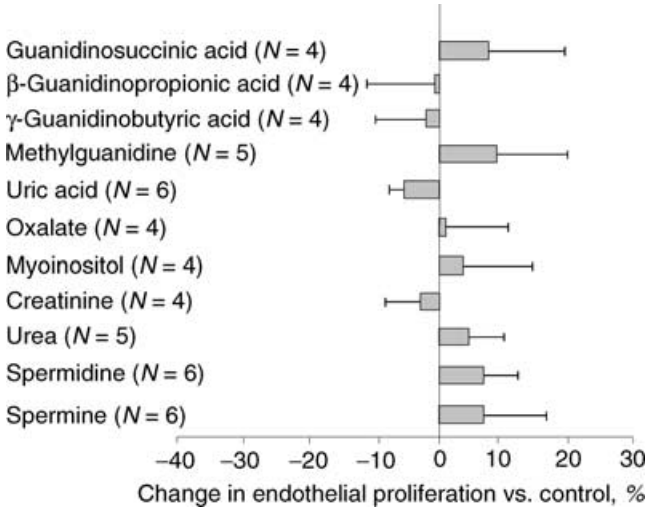

Fig. 1. Effect of free water-soluble uremic solutes on endothelial cell proliferation. Endothelial cells were incubated in medium without and with free water-soluble solutes at mean uremic concentrations. After incubation, endothelial cell proliferation was measured by enzyme-linked immunosorbent assay (ELISA) analyzing 5-bromo-2-deoxyuridine (BrdU) incorporation. Data are expressed as mean ± SEM. The number of experiments is between brackets.

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, USA). Determination of significant differences was performed by the Student paired *t* test or by analysis of variance (ANOVA) followed by a Bonferroni test. A *P* value lower than 0.05 was considered significant.

RESULTS

Effect of uremic retention solutes on endothelial cell proliferation

We measured endothelial cell proliferation by ELISA analyzing BrdU incorporation into cellular DNA, after incubation with uremic solutes at mean uremic concentration (Table 1). Among the different free water-soluble solutes tested, none impaired endothelial proliferation (Fig. 1).

The effect of protein-bound uremic solutes was analyzed without and with HSA at concentrations found in human serum (4 g/dL). The results obtained for protein-bound uremic solutes are shown in Figure 2. Among the different protein-bound solutes tested, p-cresol and indoxyl sulfate inhibited endothelial proliferation. Without HSA (Fig. 2A), p-cresol and indoxyl sulfate inhibited

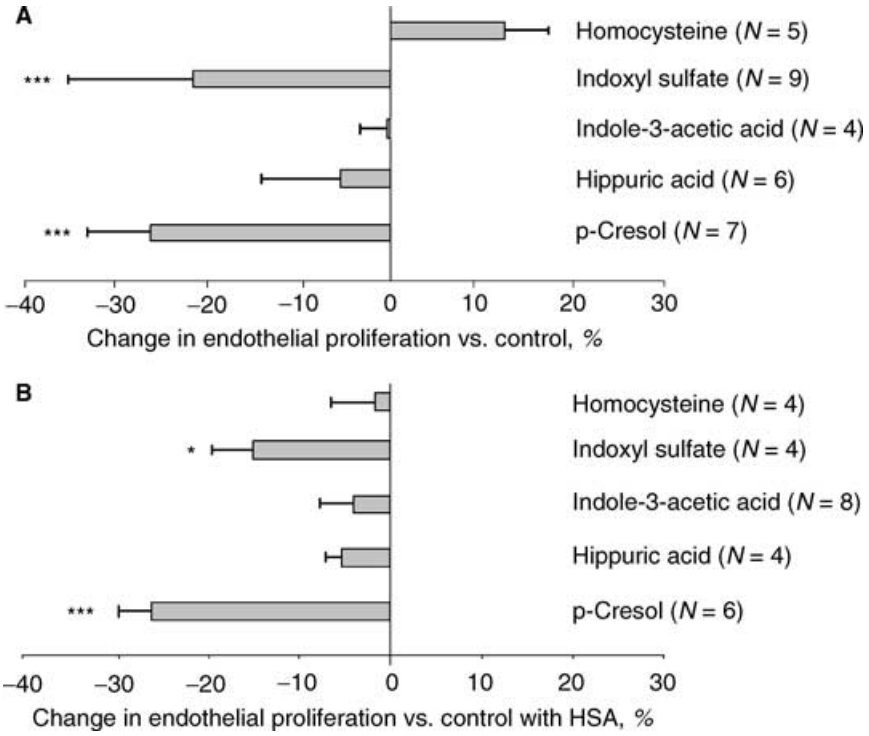

Fig. 2. Effect of protein-bound uremic solutes on endothelial cell proliferation. Endothelial cells were incubated with protein-bound solutes at mean uremic concentrations or with control medium, without (*A*) or with (*B*) of 4% human serum albumin (HSA). After incubation, endothelial cell proliferation was measured by enzyme-linked immunosorbent assay (ELISA) analyzing 5-bromo-2-deoxy-uridine (BrdU) incorporation. Data are expressed as mean ± SEM of change in proliferation versus control. The number of experiments is between brackets. Determination of significant differences versus control was performed by the Student paired *t* test. $^{*}P < 0.05$ vs. control; $^{***}P < 0.001$ vs. control.

endothelial proliferation by 26% ($P < 0.001$ vs. control) and 21% ($P < 0.001$ vs. control), respectively. With HSA (Fig. 2B), the inhibition of endothelial proliferation induced by p-cresol and indoxyl sulfate was 26% ($P < 0.001$ vs. control) and 15% ($P < 0.05$ vs. control), respectively (Fig. 2B).

To investigate whether their diluents would mask a possible effect of the solutes tested, we analyzed the effect of the different diluents (methanol, Tris, ammonium hydroxide, and water) on endothelial proliferation. No difference in endothelial proliferation was noted between cells incubated with diluents and cells incubated with culture medium alone (Fig. 3).

We performed dose-response experiments analyzing the effect of p-cresol (Fig. 4) and indoxyl sulfate (Fig. 5) on endothelial proliferation, without and with HSA. Without HSA, p-cresol at 10 μg/mL, 25 μg/mL, and 50 μg/mL induced a decrease in endothelial cell proliferation of 21% ($P < 0.001$ vs. control), 38% ($P < 0.001$ vs. control), and 54% ($P < 0.001$ vs. control), respectively (Fig. 4A), compared to cells exposed to control medium. The decrease in endothelial proliferation induced by p-cresol was dose-dependent (Fig. 4). With HSA, p-cresol inhibited endothelial cell proliferation, in the same way as without HSA (Fig. 4B).

Without HSA, all concentrations of indoxyl sulfate (25 μg/mL, 50 μg/mL, 125 μg/mL, and 250 μg/mL) inhibited endothelial proliferation relative to control medium, but the inhibition was not dose-dependent (Fig. 5A). With HSA, all concentrations of indoxyl sulfate inhibited endothelial proliferation, but this inhibition was less pronounced than without HSA (Fig. 5B).

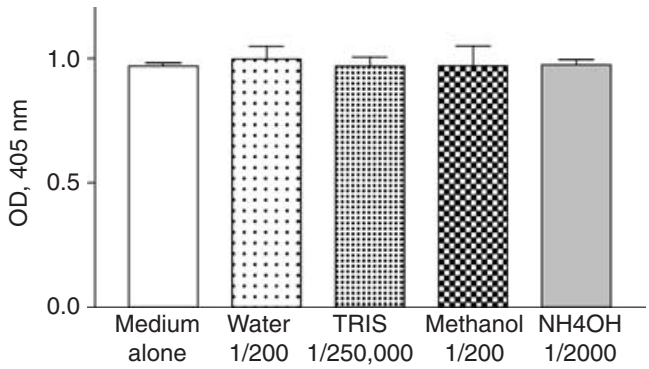

Fig. 3. Effect of diluents of uremic solutes on endothelial cell proliferation. Endothelial cells were incubated with culture medium supplemented or not with diluents of uremic solutes. After incubation, endothelial cell proliferation was measured by enzyme-linked immunosorbent assay (ELISA) analyzing 5-bromo-2-deoxy-uridine (BrdU) incorporation. Data are expressed as mean ± SEM of six independent experiments.

Effect of uremic retention solutes on endothelial cell viability

To investigate whether uremic retention solutes would affect endothelial viability, endothelial cells were incubated during 24 hours with uremic solutes at mean uremic concentration or with control mediums. In addition, for p-cresol and indoxyl sulfate, a dose-response curve

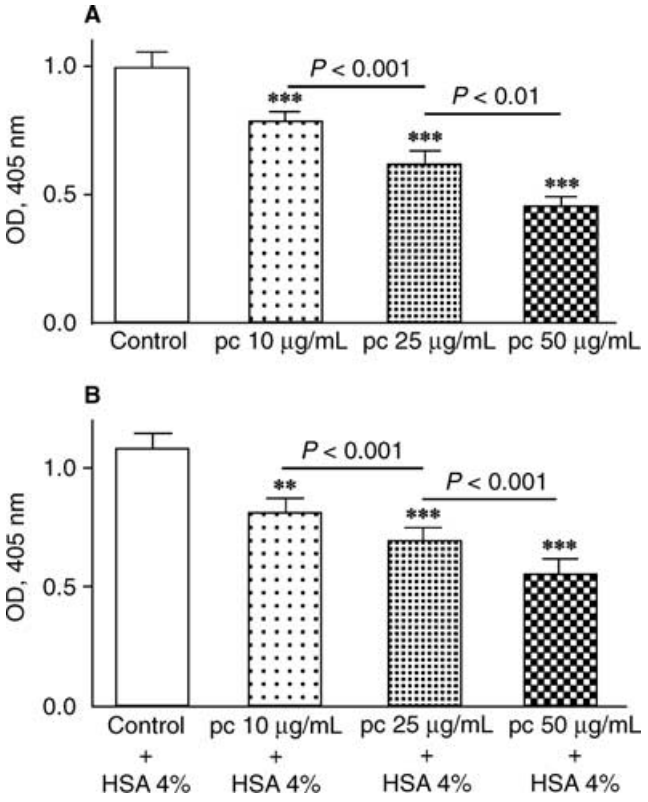

Fig. 4. Effect of p-cresol (pc) on endothelial cell proliferation. Endothelial cells were incubated in medium without and with different concentrations of p-cresol. Experiments were undertaken without (*A*) and with (*B*) 4% human serum albumin (HSA) added to the medium. After incubation, endothelial cell proliferation was measured by enzyme-linked immunosorbent assay (ELISA) analyzing 5-bromo-2-deoxy-uridine (BrdU) incorporation. Data are expressed as mean ± SEM of seven (A) or ten (B) independent experiments. Determination of significant differences was performed by analyis of variance (ANOVA) followed by a Bonferroni's multiple comparison test. $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs control.

was obtained: HUVEC were incubated with p-cresol at 10 μg/mL, 25 μg/mL, and 50 μg/mL and with indoxyl sulfate at 25 μg/mL, 50 μg/mL, 125 μg/mL, and 250 μg/mL. After 24 hours, endothelial cell viability was not impaired by the different uremic solutes tested, whatever the dose used (data not shown).

Effect of p-cresol and indoxyl sulfate on endothelial cell apoptosis

To investigate whether p-cresol and indoxyl sulfate would induce endothelial cell apoptosis, we measured their effect on the percentage of annexin V–positive cells. This percentage was not increased after incubation with p-cresol and indoxyl sulfate (Table 2), showing that these two solutes did not induce endothelial apoptosis. As expected, cells treated with TNF and cycloheximide, used as a positive control of endothelial apoptosis, displayed a strong increase in annexin V staining (Table 2).

Effect of p-cresol and indoxyl sulfate on endothelial wound repair

Since p-cresol and indoxyl sulfate inhibited endothelial proliferation, we evaluated whether these two compounds would affect endothelial wound repair. Endothelial monolayers were wounded, and wound repair was analyzed after incubation with p-cresol and indoxyl sulfate at different concentrations, without and with HSA. Without HSA, endothelial wound repair in monolayers exposed to p-cresol was significantly lower than in cells exposed to control medium (Figs. 6 and 7A). P-cresol at 10 μg/mL, 25 μg/mL, and 50 μg/mL reduced endothelial wound repair by 19% ($P < 0.01$ vs. control), 28% ($P < 0.001$ vs. control), and 40% ($P < 0.001$ vs. control), respectively (Fig. 7A). With HSA, only p-cresol at 50 μg/mL significantly inhibited endothelial wound repair (Fig. 7B). Without HSA, indoxyl sulfate at concentrations of 125 μg/mL and 250 μg/mL reduced endothelial wound repair (Fig. 8A). Endothelial wound repair was inhibited by 22% ($P < 0.01$ vs. control) and by 33% ($P < 0.001$ vs. control) by indoxyl sulfate, respectively, at 125 μg/mL and 250 μg/mL. With HSA, indoxyl sulfate decreased endothelial wound repair, but to a lesser extent than without HSA (Fig. 8B).

DISCUSSION

The dysfunction of the endothelium plays a key role in the development of cardiovascular diseases [10], which account for about 50% of mortality in CRF patients [11]. Endothelium dysfunction has been clearly demonstrated in CRF patients [1, 4, 5, 26]; however, the mechanisms involved are still obscure. In CRF patients, endothelial cells are in permanent contact with uremic retention solutes and could be a privileged target of these solutes. Endothelial cell dysfunction therefore seems to be an adequate gauge for selecting potentially harmful uremic solutes. In this way, the present study was designed to investigate the short-term effect of uremic solutes on endothelial proliferation in vitro in order to determine the solutes with the most harmful effect. The solutes were tested at uremic concentrations and under identical conditions. We showed that two solutes, p-cresol and indoxyl sulfate, at concentrations commonly found in uremia, induced an inhibition of endothelial proliferation. The inhibition of endothelial proliferation induced by p-cresol was dose-dependent. On the contrary, the inhibition of endothelial proliferation induced by indoxyl sulfate was not dose-dependent. This result suggests indoxyl sulfate has plateau effect at the concentration range used in this study. It does not exclude that higher concentrations of indoxyl sulfate might have a dose-dependent effect. However, concentrations higher than those tested in this study (i.e., much higher than those in uremic patients) should be considered nonrelevant to the in vivo situation.

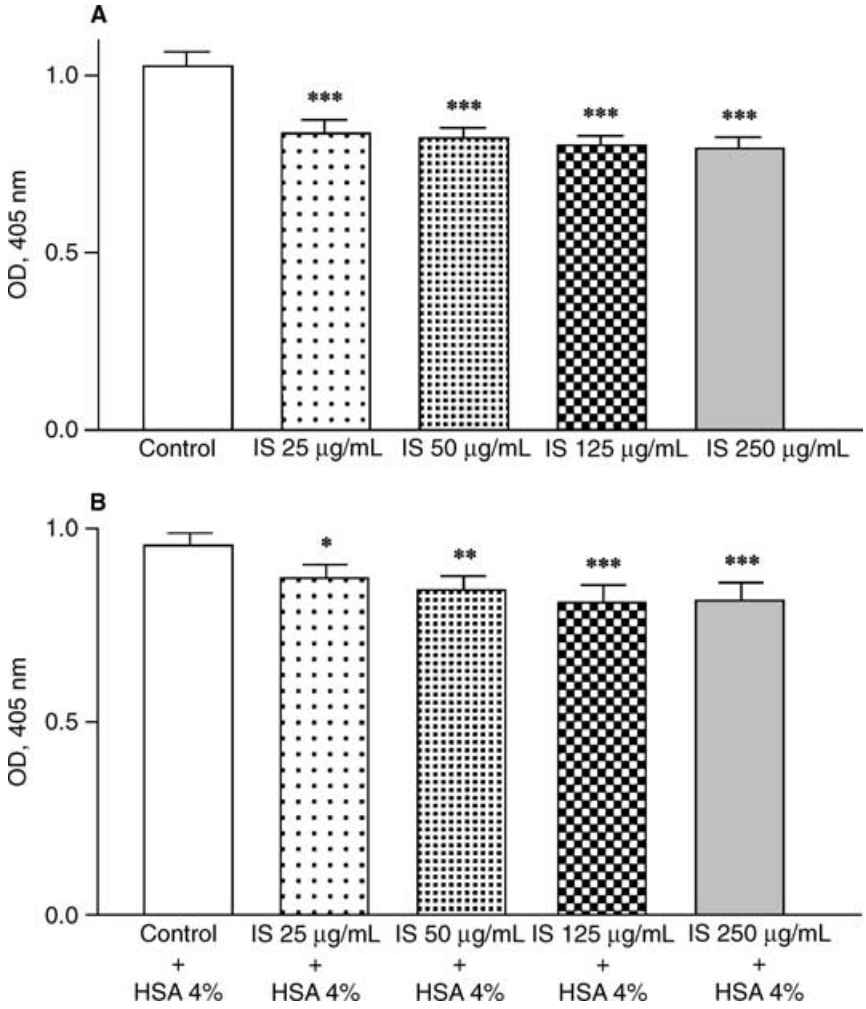

Fig. 5. Effect of indoxyl sulfate (IS) on endothelial cell proliferation. Endothelial cells were incubated in medium without and with increasing concentration of indoxyl sulfate. Experiments were undertaken without (*A*) and with (*B*) 4% human serum albumin (HSA) added to the medium. After incubation, endothelial cell proliferation was measured by enzyme-linked immunosorbent assay (ELISA) analyzing 5-bromo-2-deoxyuridine (BrdU) incorporation. Data are expressed as mean ± SEM of five (A) or seven (B) independent experiments. Determination of significant differences was performed by analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control.

Table 2. Effect of p-cresol and indoxyl sulfate on endothelial cell apoptosis

	% annexin V–positive cells
Control (methanol)	3.6 ± 0.6
p-cresol 10 μg/mL	3.4 ± 0.4
p-cresol 25 μg/mL	4.3 ± 1.0
p-cresol 50 μg/mL	4.5 ± 0.5
Control (water)	3.8 ± 0.8
Indoxyl sulfate 25 μg/mL	4.2 ± 1.6
Indoxyl sulfate 50 μg/mL	4.3 ± 0.8
Indoxyl sulfate 125 μg/mL	3.7 ± 0.5
Indoxyl sulfate 250 μg/mL	4.4 ± 1.2
Tumor necrois factor/cycloheximide	33.2 ± 2.9

Endothelial cells were incubated during 24 hours in medium without and with different concentrations of p-cresol, or in medium without and with different concentrations of indoxyl sulfate. Cells incubated with tumor necrosis factor (TNF) and cycloheximide during 24 hours were used as a positive control of apoptosis. After incubation, the percentage of annexin V–positive endothelial cells was determined by flow cytometry. Data are expressed as mean ± SEM of five independent experiments.

The other uremic solutes tested (urea, creatinine, myoinositol, oxalate, uric acid, γ-guanidinobutyric acid, β-guanidinopropionic acid, guanidinosuccinic acid, methylguanidine, spermine, spermidine, hippuric acid, indole-3-acetic acid, and homocysteine) had no significant effect on endothelial proliferation. We cannot exclude the possibility that solutes having no effect in the present study may have an effect in studies performed with longer times of incubation or higher solute concentrations. Indeed, oxalate at uremic concentration inhibits endothelial cell replication, but this was observed after more than 10 days of incubation [27]. In addition, homocysteine inhibits endothelial cell proliferation but at higher concentrations than those encountered in uremia [28, 29].

P-cresol (4-methylphenol) and indoxyl sulfate are protein-bound uremic solutes. P-cresol is an end product of protein catabolism produced by intestinal bacteria as a metabolite of tyrosine and phenylalanine [30]. Indoxyl sulfate is metabolized by the liver from indole produced by the intestinal flora as a metabolite of tryptophan [31]. In the uremic rat, indoxyl sulfate accelerates glomerular sclerosis [32] and its accumulation promotes the progression of CRF [33]. In CRF patients, serum levels of p-cresol and indoxyl sulfate are increased about 10-fold [34] and 50-fold [24], respectively. In healthy subjects, the protein

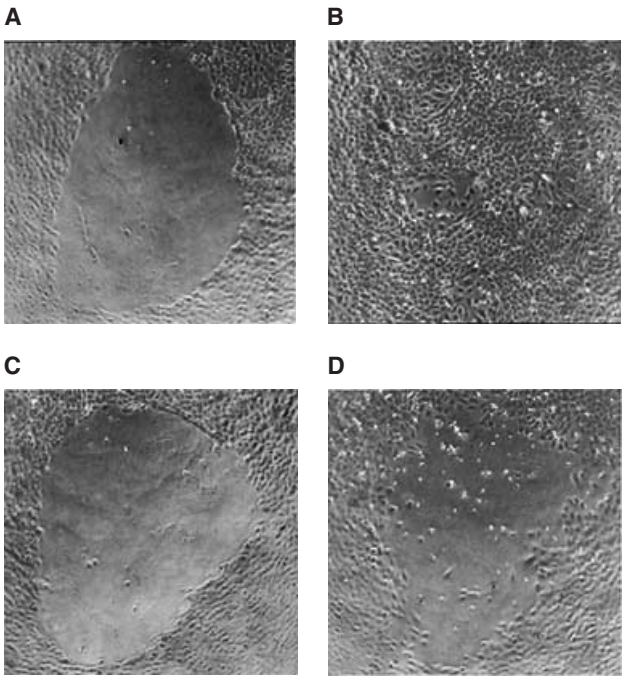

Fig. 6. Pictures of endothelial wound repair in presence of p-cresol. Endothelial monolayers were wounded, incubated with control medium or with p-cresol, and endothelial wound repair was analyzed under videomicroscopy. Pictures of wounds before addition of control medium (*A*) or p-cresol (*C*). Pictures of wounds after a 24-hour incubation with control medium (*B*) or with p-cresol at 50 μg/mL (*D*). In presence of control medium, the original wound was almost completely repaired after 24 hours of incubation (A and B). In presence of p-cresol, wound repair was inhibited (C and D).

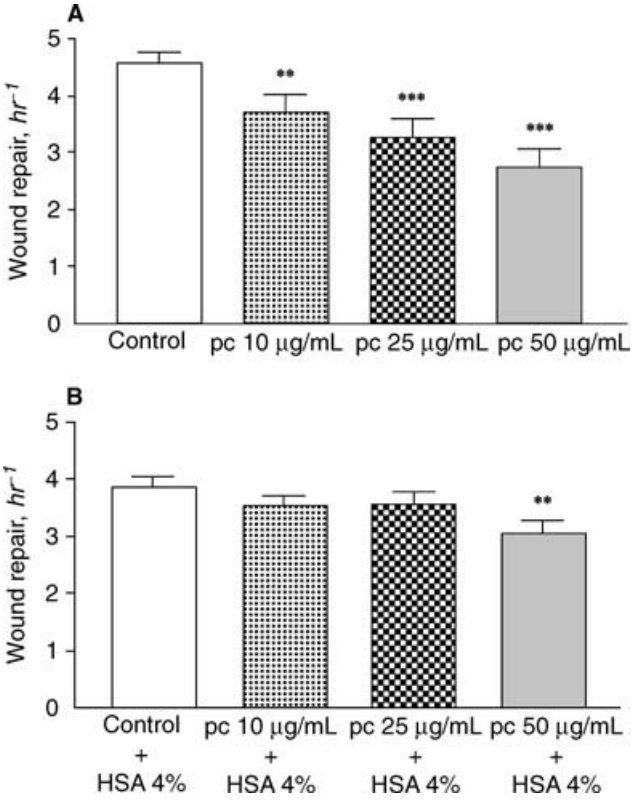

Fig. 7. Effect of p-cresol (pc) on repair of wounded endothelial monolayers. Endothelial monolayers were wounded and incubated with increasing concentrations of p-cresol. Experiments were undertaken without (*A*) and with (*B*) 4% human serum albumin (HSA) added to the medium. Endothelial wound repair was analyzed under videomicroscopy. Data are expressed as mean ± SEM of six independent experiments. Determination of significant differences was performed by analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test. $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control.

binding of these solutes is close to 100% [24, 34]. In CRF patients, approximately 90% of p-cresol [34] and 85% of indoxyl sulfate [24] are protein-bound.

In the present study, p-cresol and indoxyl sulfate decreased endothelial repair after wounding in vitro. The healing of endothelial injury requires an active repair process, closely related to the endothelial capacity to proliferate and to migrate. When endothelial integrity is disrupted, the cytoskeletal systems are activated to regulate cell migration and proliferation, in order to reestablish endothelial integrity [12]. In resting confluent endothelial cells, the actin cytoskeleton is organized as a dense peripheral band of actin microfilaments and as central microfilaments [35]. After injury, the dense peripheral band of actin microfilaments is reduced, forward actin-based lamellipodia extrusions are formed, and cells are elongated [36]. The presence and the reorganization of central microfilaments, associated with lateral cell spreading, lead to cell migration and proliferation [36].

The mechanisms by which p-cresol and indoxyl sulfate inhibit endothelial proliferation and wound repair are unknown. The inhibition observed in the present study was not attributable to cellular death because p-cresol and indoxyl sulfate did not affect endothelial viability and did not induce endothelial apoptosis. We have previously shown that p-cresol modifies the actin cytoskeleton organization in endothelial cells [abstract; Cerini C et al, *J Am Soc Nephrol* 12: 810, 2001]. We can therefore suppose that its inhibitory effect on endothelial proliferation and wound repair could be partly related to its effects on the endothelial actin cytoskeleton. To our knowledge, there is no information on the effect of indoxyl sulfate on endothelial cells. This study is the first to demonstrate such an effect, and further studies are necessary to understand the molecular mechanism of indoxyl sulfate action on endothelial cells.

Since p-cresol and indoxyl sulfate are protein-bound in plasma, we supplemented the culture medium with human albumin at concentrations found in human plasma. Without albumin, indoxyl sulfate inhibited endothelial proliferation and wound repair. A similar result was observed with albumin, but at a lower level of significance. The presence of albumin did not affect the inhibitory

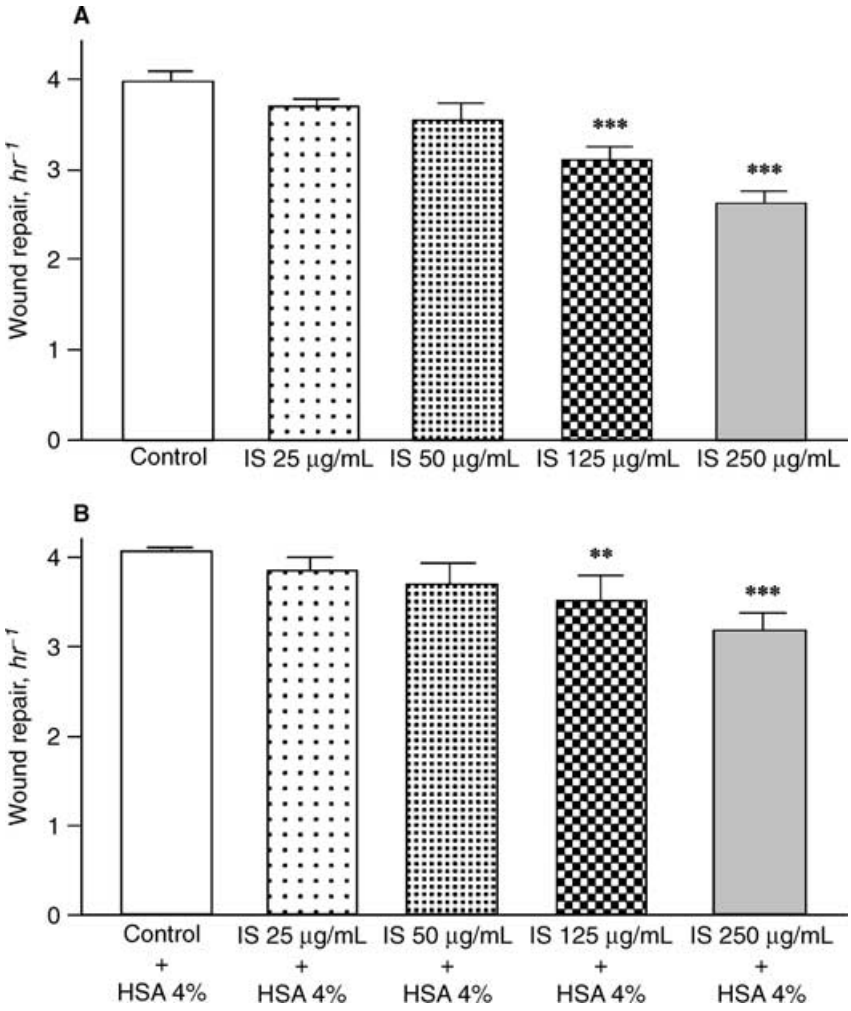

Fig. 8. Effect of indoxyl sulfate (IS) on repair of wounded endothelial monolayers. Endothelial monolayers were wounded and incubated with increasing concentrations of indoxyl sulfate. Experiments were undertaken without (*A*) and with (*B*) 4% human serum albumin (HSA) added to the medium. Endothelial wound repair was analyzed under videomicroscopy. Data are expressed as mean ± SEM of six independent experiments. Determination of significant differences was performed by analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test. $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control.

effect of p-cresol on endothelial proliferation, but it decreased its inhibitory effect on wound repair. These differences could be explained by the decrease in the free nonprotein-bound fraction of these solutes in medium supplemented with albumin. The results obtained with p-cresol suggest the free fraction of p-cresol has different impacts on endothelial wound repair and on endothelial proliferation.

That two protein-bound uremic solutes induced endothelial dysfunction in vitro is of importance because the removal of protein-bound uremic solutes by dialysis therapy is unsatisfactory. Only 30% of p-cresol and indoxyl sulfate is removed by hemodialysis therapies, which eliminate over 70% of urea and creatinine [15]. Other solutions should be developed to remove protein-bound uremic solutes like p-cresol and indoxyl sulfate. An alternative solution is to reduce the intestinal load of these solutes. A low-protein diet decreases serum levels and daily urinary excretion of p-cresol [37] and indoxyl sulfate [38]. In addition, the oral sorbent AST-120 decreases p-cresol and indoxyl sulfate levels in the uremic rat and in uremic patients [39–42], and delays the deterioration of renal function in patients [41, 42].

An increasing pathophysiologic role has been attributed recently to the protein-bound uremic solutes [43], of which at least 20 different moieties are known at present [44]. Among those, the most important biochemical/biological effects can be summarized as follows: p-cresol, inhibition of leukocyte function [45, 7], neuronal cell dysfunction [46]; indoxyl sulfate, decline in residual renal function [47], neuronal dysfunction [46]; homocysteine, vascular disease [48]. A more extended summary of the pathophysiologic role of the protein-bound uremic solutes can be found in review publications [30, 31, 43]. Until recently, there was no evidence that the protein-bound uremic solutes had a clinical impact. De Smet et al [49], however, demonstrated a link between hospitalization rate and hospitalization because of infection in one hand, and free plasma p-cresol levels on the other.

CONCLUSION

We showed that two protein-bound uremic solutes, p-cresol and indoxyl sulfate, inhibit endothelial cell proliferation and wound repair in vitro. These protein-bound solutes are poorly removed by hemodialysis therapies. In addition, endothelial cells from patients are chronically exposed to these solutes, so one can suppose that the in vivo effects of p-cresol and indoxyl sulfate may be even more prominent in patients. This study suggests that these solutes may participate in endothelial dysfunction in CRF patients.

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