A Uremic Solute, P-Cresol, Inhibits the Proliferation of Endothelial Progenitor Cells via the p38 Pathway

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Background: Endothelial dysfunction is a consistent finding in uremic patients. Whether the uremic solutes, p-cresol and indoxyl sulfate, affect the cellular function of endothelial progenitor cells (EPCs) was tested.

Methods and Results: EPCs were isolated from healthy adults and treated with p-cresol (10–80 μg/ml) or indoxyl sulfate (25–200 μg/ml) with ranges of concentration similar to those found in uremic patients. The effect of p-cresol or indoxyl sulfate on the viability of EPCs was examined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In vitro angiogenesis of EPCs was tested by a matrigel assay. Signal pathways activated by these solutes were also studied. The viability of EPCs was dose- and time- dependently inhibited by p-cresol and indoxyl sulfate, respectively (both P<0.05). The angiogenesis capacity of EPCs was suppressed significantly by p-cresol but not by indoxyl sulfate. Phosphorylated p38 and Erk1/2 was increased by p-cresol, while P38 inhibitor SB203580 reversed the effect of p-cresol in the MTT assay. Notably, a dose of 80 μg/ml p-cresol decreased the Notch1 intracellular domain level in EPCs.

Conclusions: This study has demonstrated that p-cresol inhibits proliferation of EPCs via activation of p38 MAPK pathways. P-cresol also attenuates angiogenesis function of EPCs and interferes with the Notch1 pathway. (Circ J 2011; 75: 2252–2259)

Key Words: Endothelial progenitor cell; Indoxyl sulfate; P-cresol

Cardiovascular diseases are the leading causes of mortality in uremic patients.1 Besides common cardiovascular risk factors, circulatory pathological substances including uremic solutes, asymmetric dimethylarginine and reactive oxygen species might also play a role in the pathogenic processes of cardiovascular diseases.2 Aggravated endothelial dysfunction has been uncovered in almost all uremic patients. However, information regarding the impairment of the vascular endothelium in uremic milieu remains limited. The pathological effects of the uremic solutes and the mechanisms of these solutes contributing to endothelial dysfunction are largely unclear.

Recent studies have shown that endothelial progenitor cells (EPCs) have great potential for re-endothelialization and cell therapy. This group of cells, derived from bone marrow, might circulate in peripheral blood and repair the injured endothelium. It has been suggested that some uremic solutes might inhibit the function of EPCs. Homocysteine and advanced glycation end products could depress the function of EPCs in vitro.3,4 P-cresol and indoxyl sulfate are unique solutes that are retained in uremic patients. Some studies had shown that p-cresol and indoxyl sulfate could inhibit proliferation of endothelial cells in vitro,5 but there is no information revealing the effect of p-cresol and indoxyl sulfate on EPCs.

Intracellular signal transduction pathways including the mitogen-activated protein kinase (MAPK) pathway and Notch pathway are crucial for the physiological function of EPCs. The P38 pathway could downregulate EPCs.6 The Notch pathway is closely involved in the development and growth of EPCs. Thus, the present study investigated whether the uremic solutes, p-cresol and indoxyl sulfate, affect the cellular function of EPCs. We further examined whether the activity of the MAPK and Notch pathways is altered by these solutes.

Methods

Culture of Peripheral Blood and Late Outgrowth EPC

EPCs culture was performed as previously described.7 Briefly, mononuclear cells were isolated by density gradient centrifugation with Histopaque-1077 (Sigma, St. Louis, MO, USA) from 20 ml of peripheral blood from healthy donors.
P-Cresol Inhibits Proliferation of EPCs

(n=4), and 1×10^7 mononuclear cells were plated on 6-well culture dishes coated with human fibronectin and were grown in endothelial basal medium supplemented with endothelial growth medium SingleQuots (Clonetics, Walkersville, MD, USA) and 10% FCS. After 3 days in culture, non-adherent cells were removed and adherent cells were cultured for another 6–16 days. Colonies of EPC appeared between 10 and 20 days of culture. The late outgrowth EPCs at 3–5 passages were used in this experiment.

**Determination of EPC**

Confirmation of EPCs was determined, as previously described. After dilution of 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-ac-LDL) (Invitrogen, OR, USA) in EBM2 medium, cells were washed twice and incubated for 1 h at 37°C in EBM2 medium containing 2.4 μg/ml Dil-ac-LDL. After washing, cells were then fixed with 2% paraformaldehyde for 10 min and incubated with 10 μg/ml FITC-labeled Ulex europaeus agglutinin-1 (UEA-1) (Sigma) for 1 h. Only double-positive (Dil-ac-LDL and UEA-1) cells were deemed as late outgrowth EPCs.

Fluorescence-activated cell sorting (FACS) detection of EPCs was performed on late outgrowth EPCs. After 20 days in culture, EPCs (2×10^5) were incubated with phycoerythrin-conjugated monoclonal antibodies against CD34 (Santa Cruz, CA, USA), FITC-conjugated monoclonal antibodies against CD31 and CD45 (Abcam, Cambridge, UK), Alexa Fluor 647-conjugated antibodies against VEGFR2 (Biolegend, San Diego, CA, USA) and CD133 (Abcam) respectively. Quantitative FACS was performed on a FACS cytometer.

**EPCs Proliferation Assay**

The effect of p-cresol or indoxyl sulfate on EPCs proliferation was determined by a MTT assay. The range of concentrations for p-cresol and indoxyl sulfate usage was similar to those reported in uremic patients (for p-cresol, 10 μg/ml, 20 μg/ml, 40 μg/ml and 80 μg/ml; and for indoxyl sulfate, 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml being used). Control groups received a dilution of methanol equivalent to p-cresol at the highest concentration and a dilution of water equivalent to indoxyl sulfate at the highest concentration. After being cultured for 24 h, 72 h and 120 h, EPCs were supplemented with MTT (5 g/L) and incubated for another 4 h. Then the EPCs preparation was shaken with dimethyl sulfoxide (DMSO) for 10 min, before the OD measurement at 490 nm.

**Figure 1.** Characteristics of cultured endothelial progenitor cells (EPCs). (A) Photomicrograph of cultured EPCs. EPCs were stained with both Ulex europaeus agglutinin-1 (UEA-1) fluorescein isothiocyanate (FITC) (green fluorescence) and Dil-acetylated-low-density lipoprotein (Dil-ac-LDL) (red fluorescence). Both positive cells were defined as EPCs. Original magnification was ×400. (B) Flow cytometric analysis of cultured EPCs. Cells were labelled with antibodies to CD34, CD133, VEGFR2, CD31 and CD45. Late outgrowth EPCs were deemed as CD34-positive, CD31-positive, VEGFR2-positive, CD45-negative and CD133-negative.
In Vitro Angiogenesis on Matrigel
The angiogenesis capacity of EPCs was investigated on matrigel, as previously described. Based upon MTT assay results, EPCs were incubated with an additional 80 μg/ml p-cresol and 200 μg/ml indoxyl sulfate for 72h. Control groups received an equivalent volume of methanol or water. Then, EPCs (2×10⁴ cells per well) were harvested and placed on a 96-well glass slide pre-coated with Matrigel (BD Bioscience, San Jose, CA, USA) and were stimulated with 80 μg/ml p-cresol and 200 μg/ml indoxyl sulfate. Furthermore, the 80 μg/ml p-cresol and 200 μg/ml indoxyl sulfate groups were co-treated with 1 μmol/L SB203580 (Calbiochem). After 12h of incubation, cells were examined and graded as follows: 0, separated individual cells; 1, cells begin to migrate and align; 2, capillary tubes visible, but no sprouting; 3, sprouting of new capillary tubes visible; 4, closed polygons begin to form; and 5, complex mesh-like structures develop. Experiments were performed in triplicate and 2 investigators who were blinded to the experiment protocol examined the randomly selected fields.

EPCs Apoptosis Assay
The apoptotic potential of EPCs was assessed by an annexin V/propidium iodide binding assay (Invitrogen) according to the manufacturer’s instructions. To exclude the dead cells, only annexin V-positive and propidium iodide–negative cells were counted.

Western Blot
EPCs were stimulated by 80 μg/ml p-cresol or 200 μg/ml indoxyl sulfate for 0, 15, 30 and 60min. A respective solvent control received an equivalent volume of methanol for 30min. Then the cells were lysed in a lysis buffer (RIPA), and were fractionated by SDS-PAGE on 10% polyacrylamide gels. Then, proteins were transferred to PVDF membranes. The membrane was incubated with phospho-p38, total p38, phospho-Erk1/2, total Erk1/2 antibody or Notch1 rabbit antibody (all from Cell Signaling, Danvers, MA, USA). Then, after washing, the membrane was incubated with a HRP-linked secondary antibody and detected by using an enhanced chemiluminescence kit.

Immunohistochemistry
After being incubated with 80 μg/ml p-cresol, 200 μg/ml indoxyl sulfate or an equivalent volume of methanol for 72h, EPCs were fixed with 4% formaldehyde for 10min followed by permeabilization for 10min in PBS containing 0.1% Triton X-100. After blocking, the samples were incubated with Notch1 primary antibodies (Cell signaling) at 4°C overnight, and detected by using Cy3-conjugated anti-rabbit secondary antibodies (Jackson, West Grove, PA, USA). Then, the cells were stained with 100nmol/L DAPI (4’,6-diamidino-2-phenylindole) for 5min, and mounted. Cy3-labeled EPCs were viewed under an Olympus fluorescence microscope (Olympus, Japan).

Statistical Analysis
All data are presented as mean±standard error of the mean (SEM) unless otherwise stated. Comparisons were performed by using a Student’s t-test, when appropriate. The chi-squared test was used to compare categorical variables. P values of not more than 0.05 were considered statistically significant.

Results

Characteristics of EPCs
Colonies of late outgrowth EPC appeared between 10 and 20 days of culture and were identified as a well-circumscribed monolayer of cells with a cobblestone appearance. Double-positive (Dil-ac-LDL and UEA-1) cells were deemed as late outgrowth EPCs. FACS showed that these late outgrowth EPCs were CD34-positive, CD31-positive, VEGFR2-positive, but CD45-negative and CD133-negative (Figure 1).

Effect of P-Cresol and Indoxyl Sulfate on Endothelial Cell Viability
To investigate the effect of p-cresol and indoxyl sulfate on the viability of EPCs, we performed a time- and dose-response experiment. P-cresol at concentrations of 10 μg/ml, 20 μg/ml, 40 μg/ml and 80 μg/ml induced a decreased proliferation in EPCs of 16% (P<0.05 vs. control), 19% (P<0.05 vs. control), 31% (P<0.001 vs. control) and 42% (P<0.001 vs. control), respectively, after 72h of culture (Figure 2A). Similarly, indoxyl sulfate at concentrations of 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml induced a decrease in EPCs proliferation of 10% (P<0.05 vs. control), 14% (P<0.01 vs. control), 19% (P<0.001 vs. control) and 26% (P<0.001 vs. control), respectively, after 72h of culture (Figure 2B). The effect on prolifera-
Figure 3. Effect of p-cresol and indoxyl sulfate on the angiogenesis capacity of late outgrowth endothelial progenitor cells (EPCs). (A) Representative photomicrographs of tube formation with 80 μg/ml p-cresol and 200 μg/ml indoxyl sulfate combined with or without 1 μmol/L SB203580. Original magnification was ×40. (B) Tube formation index of EPCs. P-cresol significantly inhibited the formation of tube-like structures (*P<0.05 vs. control). Data are expressed as mean±standard error of the mean of 3 independent experiments.

Figure 4. Effect of p-cresol and indoxyl sulfate on the endothelial progenitor cells (EPCs) apoptosis. No significant differences were detected by the annexin V/propidium iodide binding assay (P>0.05 vs. control).
Effect of P-Cresol and Indoxyl Sulfate on EPCs’ Angiogenesis Capacity In Vitro

Because p-cresol and indoxyl sulfate inhibited the proliferation of EPCs, we therefore investigated whether these 2 solutes affect the tube-like structure formation of EPCs. After being stimulated for 72h, p-cresol at 80 μg/ml reduced the capacity of tube-like structures forming (P<0.05 vs. control, Figure 3), however, indoxyl sulfate did not have this effect.

Effect of P-Cresol and Indoxyl Sulfate on Endothelial Cell Apoptosis

To investigate whether p-cresol and indoxyl sulfate induced endothelial cell apoptosis, we measured the percentage of annexin V–positive cells after solute stimulation. This percentage was not increased after incubation with p-cresol or indoxyl sulfate (Figure 4), indicating that these 2 solutes of tested concentrations did not induce endothelial apoptosis.
Figure 6. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed the p38 inhibitor, SB203580, partially reversed the inhibitory effect induced by p-cresol after a 24-h stimulation. Incubation with p-cresol and 1 μmol/L SB203580 induced an increase of EPCs’ viability (**P<0.01 vs. p-cresol only group, ***P<0.001 vs. p-cresol only group). This trend was not observed in the indoxyl sulfate group.

Figure 7. Effect of p-cresol and indoxyl sulfate on the Notch1 pathway. (A) Western blot showed that the Notch1 intracellular domain (NICD) was downregulated after 72h incubation with p-cresol (***P<0.001 vs. control) but not with indoxyl sulfate. (B) Immunofluorescence detected that NICD was outside of the nuclei after stimulation with 80 μg/ml p-cresol. Original magnification was ×400.
Effect of P-Cresol and Indoxyl Sulfate on the Phosphorylation of p38 and Erk1/2

We investigated the effect of p-cresol and indoxyl sulfate on the phosphorylation of p38 and Erk1/2 after 15, 30, and 60 min of stimulation. In the stimulation of p38 (80 μg/ml), the phosphorylation of p38 and Erk1/2 were increased in a time-dependent manner (Figures 5A, C), but indoxyl sulfate (200 μg/ml) did not show a similar effect (Figures 5B, D).

Effect of p38 and Erk1/2 Inhibitors on the Viability of EPCs

Furthermore, we studied the effect of SB203580 (p38 inhibitor) and PD98059 (Erk1/2 inhibitor) on the viability of EPCs. We found that SB203580 reversed the effect of p-cresol on the viability of EPCs, but indoxyl sulfate did not (Figure 6). The Erk1/2 inhibitor, PD98059, did not have this effect (data not shown).

Effects of P-Cresol and Indoxyl Sulfate on the Notch1 Pathway

The effects of p-cresol and indoxyl sulfate on the Notch1 pathway were determined by western blot and immunohistochemistry. After incubation with p-cresol for 72 h, the western blot showed that the Notch1 intracellular domain (NICD) was significantly downregulated, but this effect was not observed in indoxyl sulfate groups. Similar results were obtained from the immunohistochemistry experiment (Figure 7).

Discussion

Cardiovascular disease is the leading cause of death in renal insufficiency patients, accounting for nearly 50% mortality, which is 5- to 20-times greater than that for the general population.11-12 Previous studies have suggested that uremic solutes, p-cresol, is related to endothelial dysfunction in hemodialysis patients.13 EPCs, particularly late outgrowth EPCs, are a group of cells that actively participate in re-endothelialization and endothelial function.14-17 With the development and maturation of EPCs expansion techniques, EPCs appear to be a promising candidate for clinical cell therapy in the coming future.18 A clinical study has also observed a negative correlation of an increased uremic solute level with a decreased number of EPCs in hemodialysis patients.19 Our experiment supports this notion that p-cresol dose- and time-dependently inhibited EPCs’ viability via activation of the p38 pathway and depressed the in vitro angiogenesis of EPCs. In addition, Notch1 pathway activity was decreased, with attenuation of NICD nuclear translocation. These results indicate that uremic solutes significantly impair cellular growth and function of EPCs.

P-cresol, a phenolic volatile compound, is retained in renal failure. It’s a terminal metabolic product of tyrosine and phenylalanine produced by intestinal bacteria. Similarly, indoxyl sulfate is a metabolite of tryptophan catabolized by intestinal flora. These 2 chemicals are protein-bound, and their removal by hemodialysis is markedly less than that of urea and creatinine. Serum levels of p-cresol and indoxyl sulfate are greatly increased in patients with chronic renal failure.20 Previous studies have shown that p-cresol and indoxyl sulfate can depress endothelial proliferation and wound healing in vitro.5 These effects might reduce the function of in situ repairment of endothelial cells. Many studies have shown that EPCs are a promising group of cells that actively participate in endothelial repair in vivo. In patients with chronic kidney failure, the number and function of EPCs are decreased.19 The present study results are consistent with these results, in that uremic solutes impair EPCs function. Thus, dysfunctional EPCs might compromise the re-endothelial process and accelerate the progression of cardiovascular disease.

The mechanisms in which the proliferation and angiogenesis of EPCs are inhibited by p-cresol and indoxyl sulfate are not fully understood. Apoptosis does not contribute to the decreased viability. Our experiments showed that p-cresol activated p38 in a time-dependent manner. The p38 inhibitor, SB203580, blocked this effect and reversed the inhibition of EPCs in the MTT assay. These results suggest the inhibition effect of p-cresol on EPCs is partially through the p38 pathway. Another member of the mitogen-activated protein kinases, Erk1/2, was also activated by p-cresol, but the Erk1/2 blocker, PD98059, showed no effect on the viability of EPCs (data not showed). In contrast, the blocking of p38 or the Erk1/2 pathway did not improve the depressed angiogenesis capacity of EPCs. It suggests that deteriorated EPCs’ angiogenesis capacity was not caused by the activation of p38 or Erk1/2. Collectively, the pathological effects of uremic solutes are complicated and in many ways.

Our research also showed that the expression of Notch1 was depressed after stimulation by p-cresol. Moreover, the fluorescence results revealed that NICD, the activated part of Notch1, was largely distributed outside of the nuclei as well, compared with the control and the indoxyl sulfate groups. These changes might be relevant to the dysfunction of EPCs after stimulation by p-cresol. Recently, some studies have reported that the Notch1 pathway is crucial for EPC-mediated angiogenesis. Kwon et al reported that inactivation of specific Jagged-1-mediated Notch signals leads to less therapeutic potential for ischemic neovascularization.21 A similar result was also reported for endothelial cells.22 These evidences suggest that the Notch1 pathway might be critical for the angiogenesis of EPC.

P-cresol and indoxyl sulfate are 2 protein-bound uremic solutes that are not effectively eliminated by dialysis. Only 30% of p-cresol and indoxyl sulfate is removed by hemodialysis therapies, compared with over 70% elimination of urea and creatinine.23 Other solutions should be adopted to remove these uremic solutes. An oral sorbent, AST-120, decreased p-cresol and indoxyl sulfate levels in uremic patients.24-25 Other solutions, like curtailed protein consumption, could also decrease the serum levels of p-cresol26 and indoxyl sulfate.27 How these strategies would affect the outcomes of uremic patients is still unknown.

Study Limitations

We identified late outgrowth EPCs by morphology, immunofluorescent stain and FACS, according to previous studies.7,9,15 However, it’s still hard to purify a homogeneous group of EPCs because they undergo constant differentiation. Nevertheless, we used these late outgrowth EPCs when they were immature and still actively proliferated. Our Notch1 signal findings are tentative because we have identified an association rather than a solid cause-and-effect relationship, hence further investigation is required.

In conclusion, this study has demonstrated that p-cresol inhibits the proliferation of EPCs via the p38 pathway. Furthermore, the angiogenesis capacity of EPCs was also depressed by p-cresol. These findings revealed that p-cresol might contribute to the deteriorated re-endothelialization in chronic kidney disease.

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References