Original Article
Protein-bound P-cresol inhibits human umbilical vein endothelial cell proliferation by inducing cell cycle arrest at G_0/G_1

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Abstract: P-cresol is a typical protein-bound uremic toxin, which is retained in patients with renal failure. It is not known whether protein-bound P-cresol exhibits the toxicity in humans. This study aims to investigate the endothelial toxicity of protein-bound P-cresol. Cultured human umbilical vein endothelial cells (HUVEC) were treated with unbound or human serum albumin-bound (HSA, 4 g/dL), P-cresol (0, 20, 40, 80 μg/mL) for 24, 48, 72 h, respectively. Cell viability was determined by using cell counting kit-8 (CCK-8) assay. Cell apoptosis and cell cycle were assessed by using flow cytometry. The expression of cell cycle proteins in HUVEC were analyzed by using western blot and double immunofluorescent labeling assay. The results indicated that the viability of HUVEC was dose- and time-dependently inhibited by the protein-bound P-cresol (77.56% inhibition at 72 h, P<0.05) and unbound P-cresol (80.65% inhibition at 72 h, P<0.05). Most HUVECs were arrested at G_0/G_1 phase by both protein-bound P-cresol (79.63% inhibition at 72 h, P<0.05) and unbound P-cresol (81.27% at 72 h, P<0.05). Both protein-bound and unbound P-cresol enhanced the expression of p21Cip1 (0.62 and 0.60, both P<0.05) and suppressed the expression of cyclin D1 (0.49 and 0.53, both P<0.05) in a dose-dependent manner. In conclusion, unbound and protein-bound P-cresol inhibit the HUVEC proliferation by inducing cell cycle arrest at G_0/G_1 phase in a dose- and time-dependent manner, which associates with the up-regulation of p21Cip1 and down-regulation of cyclin D1.

Keywords: Protein bound P-cresol, human umbilical vein endothelial cells, cell cycle, p21Cip1, cyclin D1

Introduction

Chronic kidney disease (CKD) is a kind of global public health concern. The incidence of adult CKD has been as high as 10% in the whole world. Cardiovascular disease (CVD) is highly prevalent in CKD patients and is associated with the exposure to uremic toxins [1]. Cardiovascular and cerebrovascular events account for over 50% of deaths in patients with end-stage renal failure, despite adequate dialysis [2]. Why enough dialysis could not modify the incidence and mortality of cardiovascular and cerebrovascular events? Are there any toxins which can't be eliminated by the dialysis? Are there some toxins that play important role in the mechanism of cardiovascular and cerebrovascular damage? Dialysis can't efficiently

eliminate the protein-bound uremic toxins [3]. Our previous studies showed that uremic serum damages the vascular endothelial cells and results in accelerated atherosclerosis [4]. Therefore, it is important to investigate the role of protein-bound toxins in the pathogenesis of cardio- and cerebrovascular disease.

Previous studies reported that the protein-bound uremic toxins (e.g., indoxyl sulfate, homocysteine, etc.) are associated with the endothelial dysfunction in the patients with CKD, but not with the increased incidence of cardiovascular disease [5, 6]. Recent experimental studies showed significant increases in serum levels of P-cresol in the mid-to-late stage of chronic kidney disease (CKD) and were associated with cardiovascular mortality in clinical

[7-9]. Free P-cresol sulphate is a predictor for mortality in patients at different stages of chronic kidney disease [10]. Studies have mostly focused on the toxicity of unbound fraction of P-cresol. It is not known whether protein-bound P-cresol was correlated with cardiovascular diseases.

In this study, the protein-bound P-cresol and unbound P-cresol were bounded to the human serum albumin (HSA, with 4% concentration) [11, 12] to stimulate the cultured human umbilical vein endothelial cells (HUVEC), and to investigate the effects of unbound P-cresol and protein-bound P-cresol on HUVEC proliferation and cell cycle.

Materials and methods

Cell culture

HUVEC were obtained from American Type Culture Collection (ATCC, Manassas, VA). HUVEC were cultured in RPMI 1640 medium (Gibco, USA), and supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C and atmospheric conditions of 95% O_2 and 5% CO_2. The medium was replaced every two days. For experimental use, HUVEC were plated onto glass slides in medium containing 10% fetal bovine serum. At 80% confluence, HUVEC were treated with RPMI 1640 medium containing 2% fetal bovine serum for 24 h, and then treated with 20, 40, and 80 µg/ml HSA-bound P-cresol and unbound P-cresol, respectively [11, 13, 14]. Equivalent volume of methanol solvent of P-cresol) was added to the culture medium as control. After treating for 24 h, 48 h and 72 h, the cells were harvested for detection.

Reagents

P-cresol (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol (Sigma, USA) to obtain a stock solution (1600 µg/ml) and stored at -20°C. The stock solution was diluted to the concentrations of 20 µg/ml, 40 µg/ml, and 80 µg/ml by using the culture medium. During the experiment, the concentration of methanol in the medium was no more than 0.1%. The cell counting kit-8 was obtained from Dojindo Laboratories (Kumamoto, Japan). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Human serum albumin (HSA) solution was purchased from LFB (Courtaboeuf, France). The p21Cip1 antibody, p27Kip1 antibody, p15Ink4B antibody, p16Ink4A antibody, cyclin D1 antibody, CDK4 antibody, goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies and GAPDH antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Propidium iodide (PI) was obtained from Invitrogen (Carlsbad, California, USA). Protein size markers were purchased from Amersham Biosciences (Piscataway, NJ, USA). PVDF (Immobilon polyvinylidene difluoride) membranes and ECL chemiluminescence detection kit were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, England). All other reagents used were of analytical grade.

CCK-8 assay for cell viability

HUVEC were seeded into 96-well culture plates (2×10^4 cells/well, 100 µl/well) and incubated for 24 h at 37°C and atmospheric conditions of 95% O_2 and 5% CO_2. The cells were treated with various concentrations of protein-bound and unbound P-cresol (0 µg/ml, 20 µg/ml, 40 µg/ml, and 80 µg/ml) for 24 h, 48 h and 72 h, respectively. At the end of the culture, 10 µl of the CCK-8 reagent was added to each well. After 4 h of incubation at 37°C, the absorbance was determined at 450 nm using a micro-plate reader (DTX880, Beckman, Germany).

Apoptosis assay

HUVEC were seeded in 6-well plates at 2×10^4 cells/well and cultured overnight. The cells were incubated with various concentrations of protein-bound and unbound P-cresol for 72 h. At the end of the treatment, cells were collected by using trypsin without EDTA. Flow cytometry analysis of apoptotic cells was carried out by using an Annexin V-FITC/PI staining kit. After washing with cold PBS, the cells were re-suspended in binding buffer (100 mmol HEPES, pH 7.4, 100 mmol NaCl, and 25 mmol $CaCl_2$) followed by staining with Annexin V-FITC/PI at room temperature in darkness for 15 min. Apoptotic cells were then evaluated by gating PI and Annexin V-positive cells on a fluorescence activated cell-sorting (FACS) flow cytometry (BD Biosciences, San Jose, CA). All experiments were performed in triplicate.
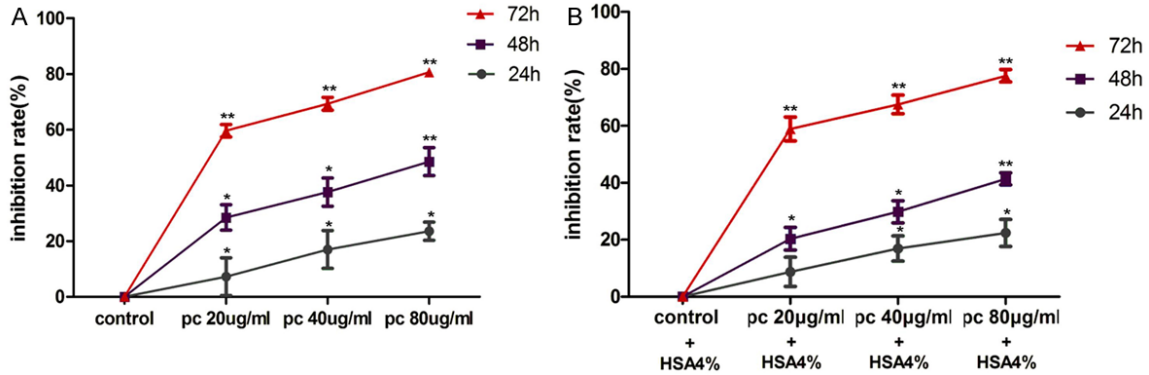
Figure 1. Effect of P-cresol on HUVEC growth inhibition. HUVEC were incubated with different concentrations of unbound (A) and protein-bound P-cresol (B) (20-80 μg/ml). Cell growth inhibition was measured using the CCK-8 assay. Results were expressed as a percentage of the control. Data were expressed as means ± SD. *P<0.05 and **P<0.01 represent the inhibition rate in protein unbound or protein-bound P-cresol group compared to the control group.

Cell cycle assay

After incubation for 72 h, cells were harvested, washed once with PBS, and fixed in 70% ethanol overnight. The cells were washed twice in PBS. Staining for DNA content was performed with 50 mg/ml propidium iodide and 1 mg/ml RNase A for 30 min. Analysis was performed on a FACS flow cytometry (BD Biosciences) with Cell Quest Pro software. Cell cycle modeling was performed with Modfit 3.0 software (Verity Software House, Topsham, ME).

Western blot

The cells were harvested, washed twice with ice-cold PBS and re-suspended in lysis buffer and sonicated briefly. After centrifugation at 12,000×g for 10 min, the supernatant was prepared as protein extract. Protein concentrations were measured by using Nucleic Acid and Protein Analyzer (Beckman DU640, USA) at 260 nm. Equal amounts of protein were separated on 12 or 15% sodium dodecyl sulfate polyacrylamide gels. The proteins were electrophoretically transferred onto polyvinylidenedifluoride (PVDF) membranes by using Bio-Rad Mini Protean II apparatus (Bio-Rad, Hercules, CA). The blots were blocked with 5% non-fat milk in PBS-T (80 mmol Na_2HPO_4, 20 mmol NaH_2PO_4, 100 mmol NaCl, and 0.1% Tween-20 at pH 7.5) for 1 h. The p21Cip1 antibody, p27-Kip1 antibody, p15Ink4B antibody, p16Ink4A antibody, CDK4 antibody, cyclin D1 and GAPDH (as control) antibodies were diluted in a blocking buffer and incubated with the blots over-

night at 4°C. Bound antibodies were then detected with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody according to the instructions provided with the ECL kit (Amersham, Franklin Lakes, NJ).

Double immunofluorescent labeling

The cells were collected and fixed in 4% paraformaldehyde for 30 min and subsequently washed twice with PBS. The cells were incubated with serum for 30 min to block non-specific binding at room temperature and rewashed with PBS. Cells were permeabilized by washing with 0.1% Triton X-100 for 20 min at 4°C, and then incubated with p21 antibody and cyclin D1 antibody overnight at 4°C. Subsequently, the cells were incubated with goat anti-rabbit or goat anti-mouse secondary antibody for 30 min at room temperature. Finally, cells were covered with a water-soluble mounting medium, and viewed with a Nikon TE2000 fluorescent microscopy (Nikon, Japan).

Statistical analysis

All data were reported as means ± standard deviation (SD). Statistical analysis was performed using the statistical package SPSS Version 18.0 for Windows (SPSS, Inc., Chicago, IL, USA). Multiple comparisons between different treatment groups were made using One-way ANOVA and significant differences between two groups were analyzed by Tukey's test. Values of *P*<0.05 were considered as statistically significant.
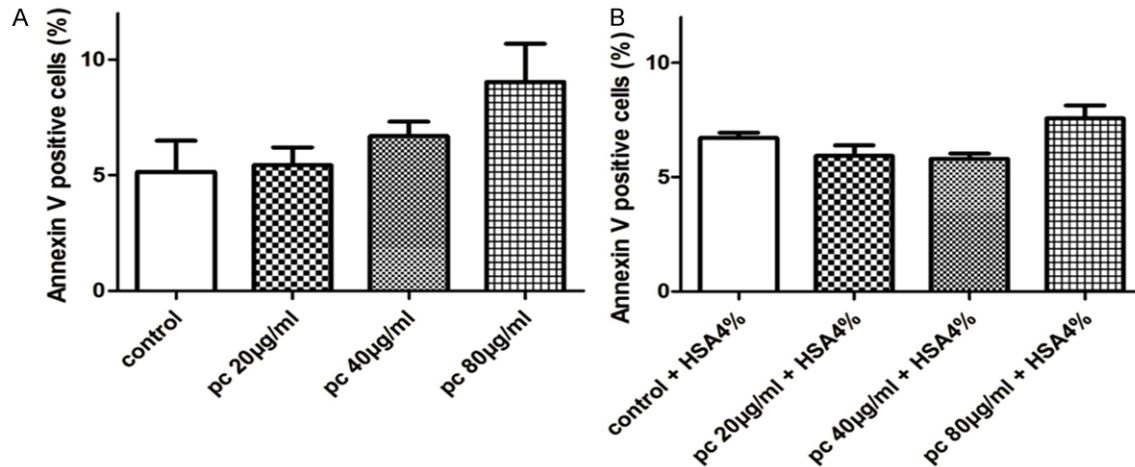
Figure 2. Effect of protein-bound and unbound P-cresol on HUVEC apoptosis. HUVEC were incubated with unbound (A) and protein-bound P-cresol (B). After incubation, the percentage of Annexin V-positive HUVEC was determined by flow cytometry. Data were expressed as means ± SD.

Results

Effect of P-cresol on cell proliferation

The effect of protein-bound and unbound P-cresol on HUVEC proliferation was shown in **Figure 1**. The 80 µg/ml unbound P-cresol induced inhibition of HUVEC proliferation by 23.58%, 48.68% and 80.65% at 24, 48 and 72 h, respectively, compared with control medium (P<0.05). The 80 µg/ml protein-bound P-cresol induced inhibition of HUVEC proliferation by 22.47%, 41.40% and 77.56%, at 24, 48 and 72 h, respectively, compared with control medium (P<0.05).

Effect of P-cresol on HUVEC apoptosis

To determine whether P-cresol induced HUVEC apoptosis, we measured the percentage of Annexin V-positive cells after solute stimulation. This percentage was not increased after incubation with protein-bound and unbound P-cresol (**Figure 2**), showing that P-cresol did not induce HUVEC apoptosis. No significant differences of unbound and protein-bound P-cresol to the control were detected (P>0.05 vs. control).

P-cresol induces HUVEC cell cycle arrest at G_0/G_1

HUVEC were arrested at G_0/G_1 by unbound and protein-bound P-cresolin a dose-dependent manner (**Figure 3**). With unbound P-cresol,

when cultured for 72 h, the G_0/G_1 phase percentage of HUVEC was significantly higher (68.15 ± 2.54% at 20 µg/ml, 75.83 ± 0.71% at 40 µg/ml, and 80.84 ± 1.61% at 80 µg/ml) compared with the control (61.64 ± 1.38%, P<0.05). With protein bound P-cresol, when cultured for 72 h, the G_0/G_1 phase percentage of HUVEC was significantly higher (69.87 ± 2.57 at 20 µg/ml, 74.88 ± 1.56% at 40 µg/ml, and 79.63 ± 1.18% at 80 µg/ml) compared with the control (62.45 ± 0.72%, P<0.05).

Effect of P-cresol on the expression of cell cycle-regulatory proteins
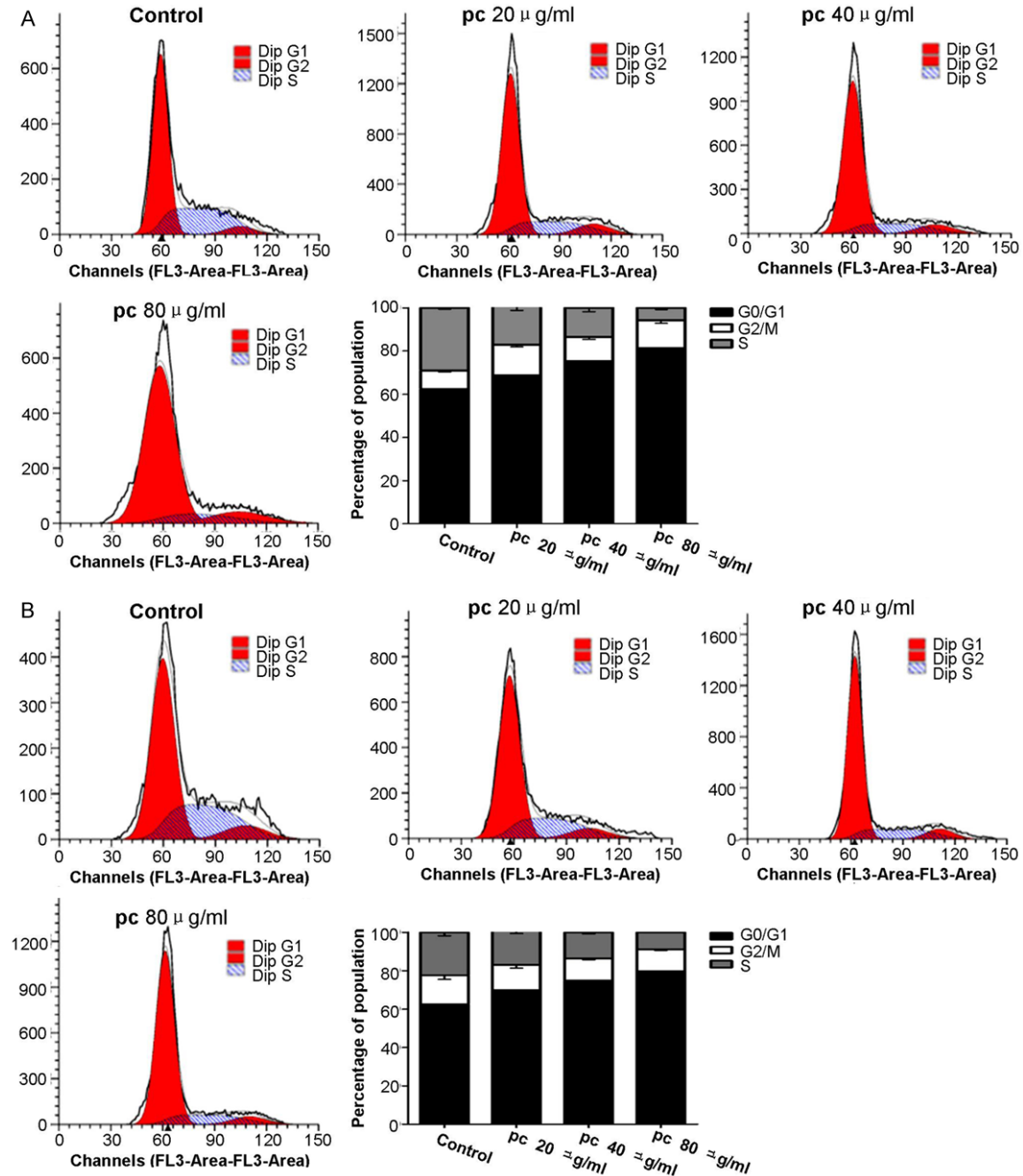
HUVEC were treated with protein-bound and unbound P-cresol (20-80 µg/ml) for 72 h, and then measured by using immuno-blotting analysis using specific antibodies. The expression results of cell cycle protein are shown in **Figures 4** and **5**. Both unbound and protein-bound P-cresol markedly diminished the expression of cyclin D1. Both unbound and protein-bound P-cresol markedly increased the expression of p21Cip1. However, CDK4, p16-INK4A, p15INK4B, and p27Kip1 levels were not affected by P-cresol treatment.

Effects of P-cresol on the expression of p21Cip1 and cyclin D1 proteins

To further evaluate the influence of P-cresol on the expression of p21Cip1 and cyclin D1 proteins, double immuno-fluorescent labeling technique was performed in HUVEC. Cultured
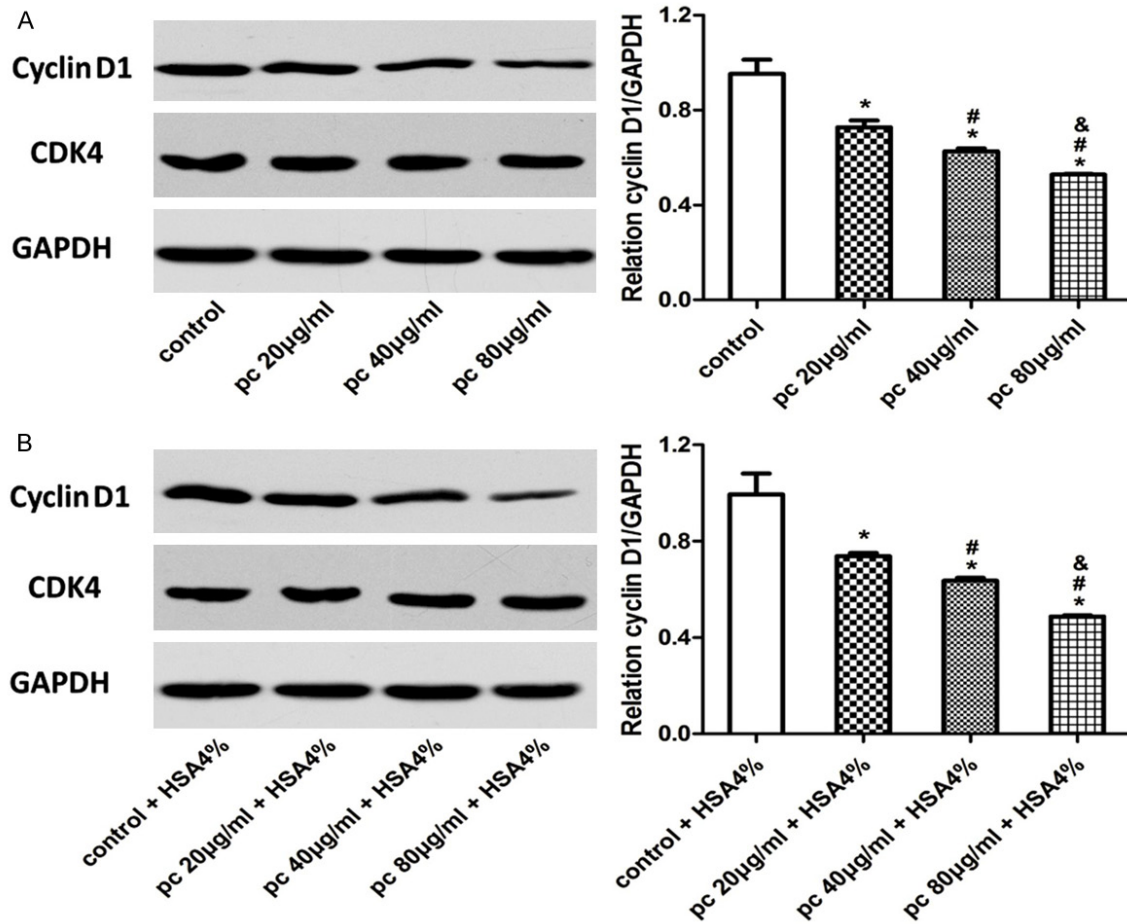
Figure 3. Effect of P-cresol on HUVEC cell cycle. HUVEC were incubated in serum-free RPMI 1640 for 24 h and treated with unbound (A) and protein-bound (B) P-cresol (20-80 µg/ml, in RPMI 1640 2% FBS) for 72 h. Cells were stained with propidium iodide and the cell cycle was analyzed by flow cytometry for DNA content. Representative cytometric profiles and percentages of each phase were shown (A and B). The program Cell Quest was used for acquisition and analysis of the FACS scans. Data were expressed as means ± SD. G_0/G_1 = cells in G_0/G_1 cell cycle phases, S = cells in S cell cycle phase, G_2/M = cells in G_2/M cell cycle phases.

cells were treated with protein-bound and unbound P-cresol (80 µg/ml) for 72 h. The results are illustrated in **Figure 6**, indicating that p21 Cip1 staining was apparently increased, while cyclin D1 staining was significantly decreased compared with the control.

Discussion

Cardiovascular disease is a frequent complication of end-stage chronic kidney disease (CKD). Endothelial dysfunction exacerbates atherosclerosis [15]. The endothelium of patients with
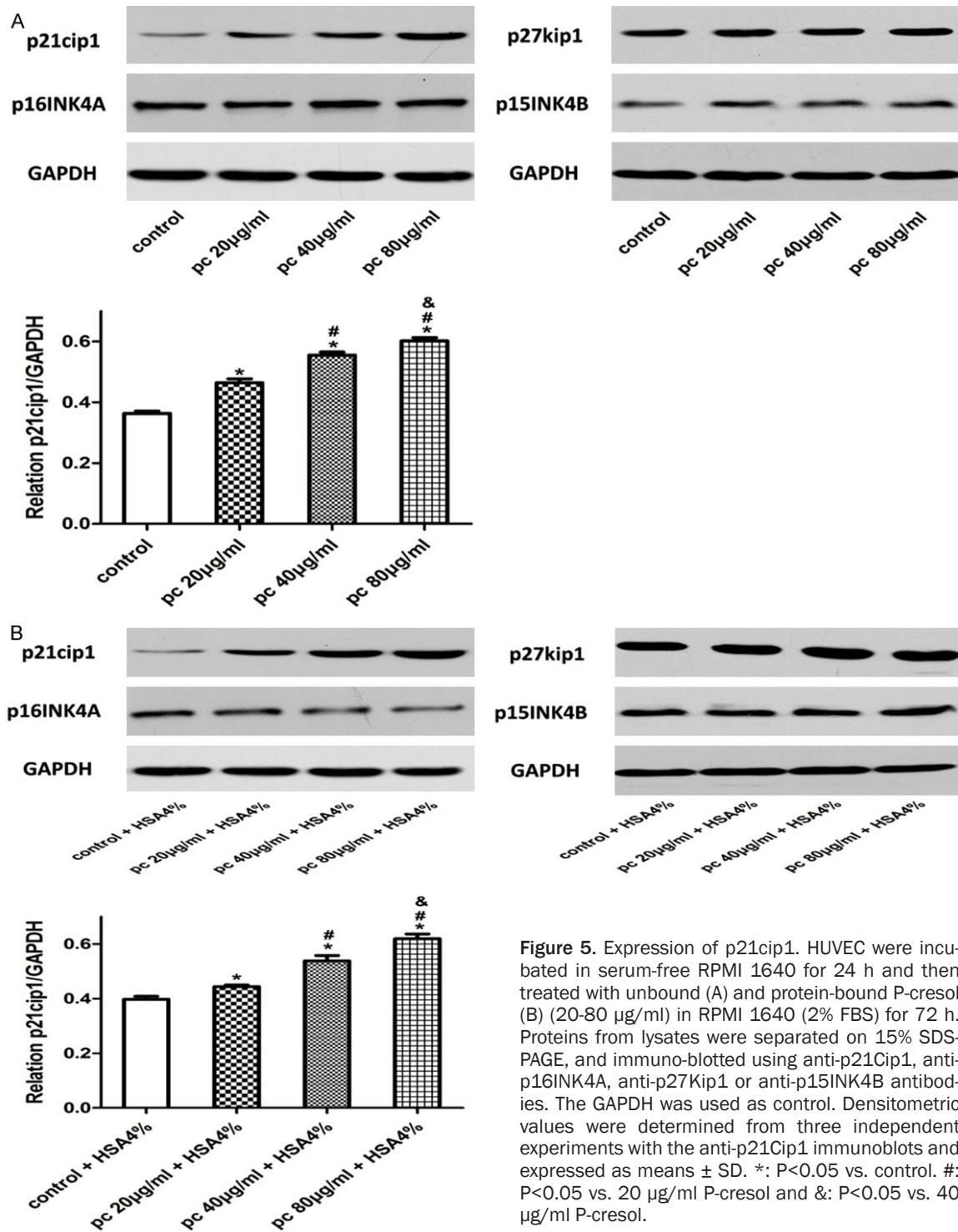
Figure 4. Expression of cyclin D1. HUVEC were incubated in serum-free RPMI 1640 for 24 h and then treated with unbound (A) and protein-bound P-cresol (B) (20-80 µg/ml) in RPMI 1640 (2% FBS) for 72 h. Proteins from lysates were separated on 15% SDS-PAGE, and immuno-blotted using anti-cyclin D1 or anti-CDK4 antibodies. The GAPDH was used as control. Densitometric values were determined from three independent experiments with the anti-cyclin D1 immuno-blots and expressed as means ± SD. *: $P<0.05$ vs. control. #: $P<0.05$ vs. 20 µg/ml P-cresol and &: $P<0.05$ vs. 40 µg/ml P-cresol.

CKD is permanently exposed to uremic toxins, which are therefore, specific pathologic agents inducing endothelial dysfunction [16]. Previous research studies have shown that P-cresol is associated with endothelial dysfunction in hemodialysis [7]. In vitro, P-cresol was shown to affect the inflammatory response by decreasing endothelial cell resistance to inflammatory cytokines [17]. P-cresol induces shedding of endothelial micro-particles in the absence of overt endothelial damage in vitro and promotes inflammation in un-stimulated leukocytes [18, 19]. These findings suggest that P-cresol may increase the risk of vascular damage in patients with renal disorders. It is not known whether protein-bound P-cresol also induced endothelial dysfunction.

P-cresol, a phenolic and volatile compound, is one of the prototypes of protein-bound uremic toxins. It originates from bacterial fermentation in the large intestine and is synthesized from the metabolism of dietary tyrosine and phenylalanine [20]. The serum levels of P-cresol in uremic patients were increased about 10-fold [21, 22]. Dialysis easily eliminated unbound P-cresol due to its low molecular weight of only 108.1D [20]. However, most (94%) P-cresol in circulation was bound to plasma protein, and therefore, can't be eliminated by dialysis [22].

Our results showed that 80 µg/ml of unbound P-cresol induced HUVEC proliferation inhibition by 80.65%, and that 80 µg/ml protein-bound P-cresol induced HUVEC proliferation inhibition

Figure 5. Expression of p21cip1. HUVEC were incubated in serum-free RPMI 1640 for 24 h and then treated with unbound (A) and protein-bound P-cresol (B) (20-80 μg/ml) in RPMI 1640 (2% FBS) for 72 h. Proteins from lysates were separated on 15% SDS-PAGE, and immuno-blotted using anti-p21Cip1, anti-p16INK4A, anti-p27Kip1 or anti-p15INK4B antibodies. The GAPDH was used as control. Densitometric values were determined from three independent experiments with the anti-p21Cip1 immunoblots and expressed as means ± SD. *: P<0.05 vs. control. #: P<0.05 vs. 20 μg/ml P-cresol and &: P<0.05 vs. 40 μg/ml P-cresol.

by 77.56% compared with the control. These data demonstrated that both unbound P-cresol and protein-bound P-cresol significantly inhibited HUVEC proliferation, in vitro. The inhibitory effect of unbound and protein-bound P-cresol on HUVEC proliferation was gradually increased with increase in the P-cresol dose and extension of the reaction time. The data indicated a dose- and time-dependent inhibition of HUVEC proliferation by both unbound and protein-bound P-cresol. In uremic patients, protein-bound P-cresol was not eliminated by dialysis,
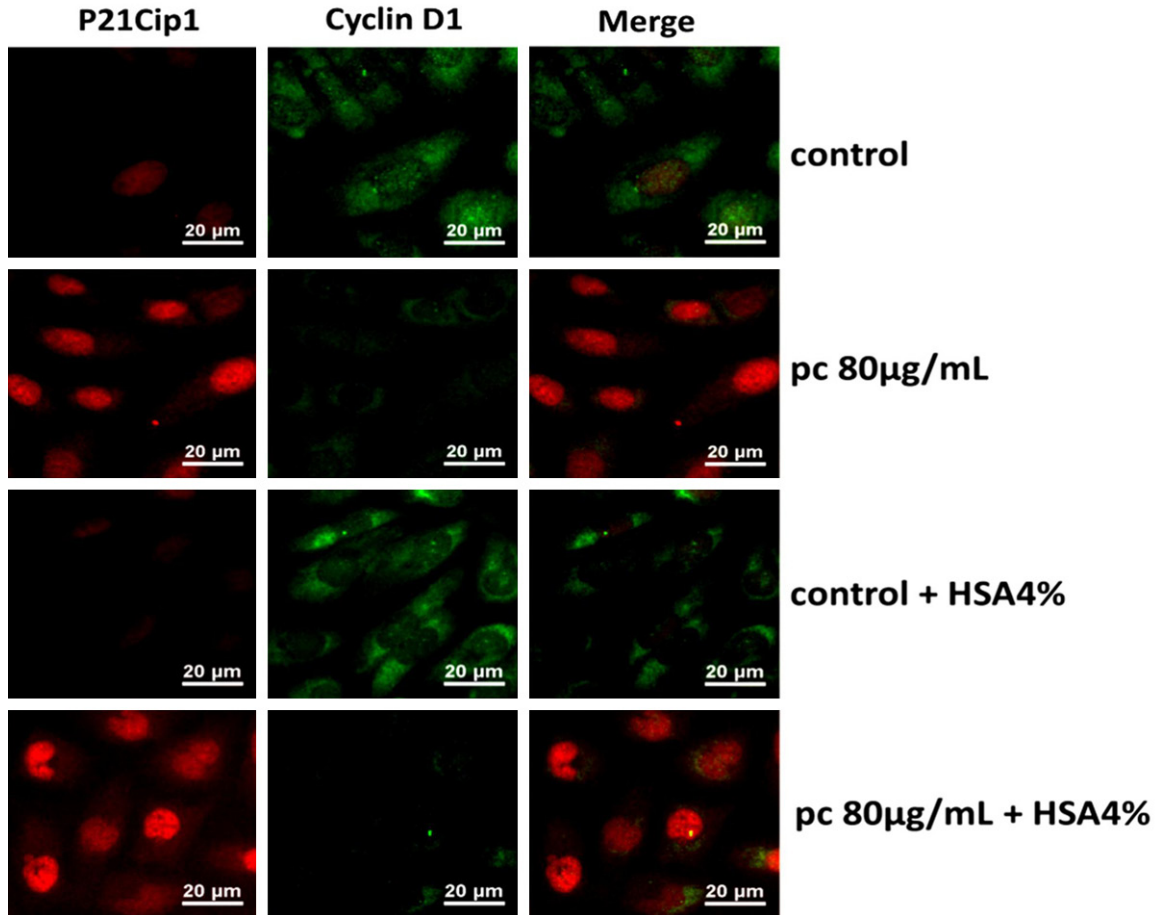
Figure 6. Effects of P-cresol on the p21Cip1 and cyclin D1 protein expression. Cells were incubated with unbound P-cresol (80 µg/ml), and protein-bound P-cresol (80 µg/ml) for 72 h. Methanol and albumin were used as controls. Representative photographs of double immuno-fluorescent labeling are shown. The red fluorescence represents p21Cip1, the green fluorescence represents cyclin D1.

resulting in ongoing accumulation in the circulation, stimulating the vascular endothelium, inhibiting endothelial proliferation and repair, which might be an important mechanism of accelerated atherosclerosis. These results were consistent with previous clinical observations that P-cresol increased cardiovascular risk in CKD patients [8].

Many toxins induce cellular apoptosis, but our data showed that P-cresol was not associated with HUVEC apoptosis. G_0/G_1 arrest of the cell cycle suggested that P-cresol did not damage the vascular endothelium through apoptosis.

Mitosis is a ubiquitous and complex process involved in the growth and proliferation of cells. The two key restriction points in the cell cycle including the G_1/S and G_2/M checkpoints maintain the orderly progression of the cell cycle and ensure proper completion of each phase before progression to the next phase [23]. The G_1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first gap phase, G_1, into the DNA synthesis phase, S. The G_2/M cell cycle checkpoint determines the cell division to ensure the integrity of DNA replication [24]. The G_1/S restriction point plays an important role in cell cycle regulation by transmitting extra-cellular and intracellular signals to cell nucleus, controlling cell proliferation [23, 25]. Most of the quiescent cells in the G_1 phase enter S phase upon stimulation by growth factors, resulting in DNA replication, and initiation of cell proliferation [26]. Our experiments showed that when unbound P-cresol or protein-bound P-cresol induced G_1 phase increase in HUVEC number, while S phase HUVEC number decreased. This result demonstrated that a number of HUVEC

were arrested in the G_1 phase. The data indicated that P-cresol inhibited endothelial cell growth by suppressing DNA synthesis. Our results differed from a recent study suggesting that P-cresol led to G_2/M arrest in endothelial progenitor cells [27]. The difference might be associated with the different cell type.

Cell proliferation depends on progression through the cell cycle. Cell cycle control is achieved through the actions of a family of cyclin-dependent protein kinases (CDKs) and cyclins that initiate phosphorylation events to allow progression through checkpoints. Cell cycle is primarily regulated by complexes containing CDKs, cyclins and cyclin-dependent kinase inhibitor (CDKI), whose inactivation leads to cell cycle arrest [28, 29]. The complexes mediating the progression of cells through the G_1 phase of cell cycle and the initiation of DNA replication include cyclin D-CDK4/CDK6 and cyclin E-CDK2 [30, 31]. Two families of CDK inhibitors (CDKIs) are critical mediators of anti-proliferative signals that arrest the cell cycle. The Cip/Kip family includes p21Cip1 (also known as WAF1, Sdi1, and CAP20), p27Kip1, and p57Kip2. The INK4 family includes p16-INK4A, p15INK4B, p18INK4C, and p19INK4D. These inhibitors negatively regulate G_1 phase progression by forming complexes with CDKs and thus preventing S phase entry [32, 33]. The p21Cip1 protein binds to and inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, and thus functions as a negative regulator of cell cycle progression at G_1 and S phases [34]. In addition to growth arrest, p21Cip1 mediates cellular senescence. Further, p21Cip1 binds to proliferating cell nuclear antigen, thereby blocking DNA synthesis. Non-proliferating cells manifest a robust expression of p21Cip1, which is primarily increased in response to extra-cellular anti-mitogenic signals inducing cell cycle arrest [35]. Cyclin always acts as the regulators of CDKs. Different cyclins exhibit distinct expression and degradation patterns, which contribute to the temporal coordination of each mitotic event. Cyclin D1 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G_1/S transition [36, 37].

Our data showed that protein-bound and unbound P-cresol increased the expression level of p21Cip1 but decreased the expression level of Cyclin D1 of cultured HUVEC in a dose-dependent manner. By contrast, protein-bound and unbound P-cresol did not affect the expression of CDK4, p27Kip1, p16INK4A, and p15INK4B. These results demonstrated that P-cresol-induced G_0/G_1 phase arrest was mediated by p21Cip1 and Cyclin D1.

In conclusion, our results suggested that both unbound and protein-bound P-cresol inhibited HUVEC proliferation in a dose- and time-dependent manner by inducing cell cycle arrest at G_0/G_1 phase, which was associated with the up-regulation of p21Cip1 expression and down-regulation of cyclin D1 expression.

Disclosure of conflict of interest

None.

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