p-cresol but not p-cresyl sulfate stimulate MCP-1 production via NF-κB p65 in human vascular smooth muscle cells

p-cresol mas não p-cresil sulfato estimulam a produção de MCP-1 via NF-κB p65 em células vasculares musculares lisas humanas

ABSTRACT

Introduction: p-cresol (PC) and p-cresyl sulfate (PCS) are responsible for many of the uremia clinical consequences, such as atherosclerosis in Chronic Kidney Disease (CKD) patients. Objectives: We investigate the in vitro impact of PC and PCS on monocyte chemoattractant protein-1 (MCP-1) expression via NF-kappa B (NF-κB) p65 in VSMC. Methods: PCS was synthesized by PC sulfation. VSMC were extracted by enzymatic digestion of umbilical cord vein and characterized by immunofluorescence against α-actin antibody. The cells were treated with PC and PCS at their normal (n), uremic (u) and maximum uremic concentrations (m). Cell viability was assayed by MTT. MCP-1 expression was investigated by ELISA in cell supernatants after toxins treatment with or without the NF-κB p65 inhibitor. Results: There was no significant difference in cell viability after toxins treatment for all concentrations tested. There was a significant increase in MCP-1 expression in cells treated with PCu and PCM (p < 0.001) and PCSn, PCSu and PCSm (p < 0.001), compared with the control. When VSMC were treated with the NF-κB p65 inhibitor plus PCu and PCM, there was a significant decrease in MCP-1 production (p < 0.005). This effect was not observed with PCS. Conclusions: VSMC are involved in atherosclerosis lesion formation and production of MCP-1, which contributes to the inflammatory response initiation. Our results suggest that PC mediates MCP-1 production in VSMC, probably through NF-κB p65 pathway, although we hypothesize that PCS acts through a different subunit pathway since NF-κB p65 inhibitor was not able to inhibit MCP-1 production.

Keywords: chemokines; chronic kidney disease; NF-kappa B; uremia.

RESUMO

Introdução: p-cresol (PC) e p-cresil sulfato (PCS) são responsáveis por muitas das consequências clínicas uremia, tais como a aterosclerose em pacientes com Doença Renal Crônica (DRC). Objetivos: No presente trabalho, investigamos in vitro o impacto de PC e PCS na expressão da quimiocina monocyte chemoattractant protein-1 (MCP-1) via NF-kappa B (NF-κB) p65 em VSMC. Métodos: O PCS foi sintetizado por sulfatação do PC. As VSMC foram extraídas por digestão enzimática da veia do cordão umbilical e caracterizadas por imunofluorescência através do anticorpo α-actina. As células foram tratadas com PC e PCS em suas concentrações normal (n), urêmica (u) e urêmica máxima (m). A viabilidade celular foi avaliada pelo ensaio de MTT. A expressão de MCP-1 foi investigada por ELISA em sobrenadantes de células após o tratamento com as toxinas, com ou sem o inibidor de NF-κB p65. Resultados: Não houve diferença significativa na viabilidade das células após o tratamento com toxinas para todas as concentrações testadas. Houve um aumento significativo na expressão de MCP-1 em células tratadas com PCu e PCM (p < 0.001) e PCSn, PCSu e PCSm (p < 0.001), em comparação com o controle. Quando as VSMC foram tratadas com o inibidor de NF-κB p65 mais PCu e PCM, houve uma diminuição significativa na produção de MCP-1 (p < 0.005). Este efeito não foi observado com PCS. Conclusões: VSMC estão envolvidas na formação da lesão aterosclerótica e produção de MCP-1, o que contribui para o início da resposta inflamatória. Os nossos resultados sugerem que a PC medeia a produção de MCP-1 em VSMC, provavelmente através da via NF-κB p65 e que PCS atue através de uma subunidade diferente da via, uma vez que o inibidor da porção p65 não foi capaz de inibir a produção de MCP-1.

Palavras-chave: quimicionas; doença renal crônica; NF-kappa B; uremia.
**INTRODUCTION**

The uremic toxin \( p \)-cresol (PC), 4-methylphenol (MW: 108.14 g/mol), is a low molecular weight uremic toxin with high affinity to proteins, originated from tyrosine and phenylalanine catabolism by intestinal microorganisms. Previous studies observed that \( p \)-cresol circulates in very low concentrations, and it is metabolized to its conjugates (\( p \)-cresyl sulfate - PCS and \( p \)-cresyl glucuronidate - PCG) by the intestinal flora, during its passage through the colon and liver mucosa.\(^1\,^2\) Thus, PCS is considered the effective toxin, due to its significantly circulated concentration and biochemical impact in the body.\(^3\)

PC accumulation results in toxic *in vitro* and *in vivo* effects with potential clinical impact, since it is a major contributor to the development of uremic cardiovascular complications.\(^4\) Dialysis therapies eliminate 70% of urea and creatinine and only 30% of PC after a 4 hours session.\(^5\,^6\) This leads to progressive accumulation of PC that has been associated with increased vascular lesions, activation of leukocytes and negative impact on clinical outcome.\(^5\) In healthy subjects, the plasma concentration of PC is approximately 0.6 mg/L, but increases significantly to 20.1 mg/L in the early stages of chronic kidney disease (CKD), and to 40.7 mg/L in advanced CKD.

The pro-inflammatory effect of PCS, measured by increased formation of free radicals produced by leukocytes, contributes to vascular injury in patients with CKD.\(^7\) It was recently demonstrated that the PCS induces the release of endothelial microparticles and circulating levels of PCS is significantly correlated with the pathogenesis of cardiovascular lesion in CKD.\(^8\) Moreover, serum levels of free and total PCS were elevated in advanced CKD stages; however only free PCS seems to be a predictor of mortality in CKD.\(^2\)

It is well known that inflammatory responses are mediated by cytokines in all stages of cardiovascular disease. Many inflammatory cells when become activated, release a variety of cytokines and chemokines, which further amplifies the process and induces the recruitment of a range of cells, including vascular smooth muscle cells (VSMC), enhancing the development of lesions in the vessel wall by stimulating the excessive production of extracellular matrix proteins.\(^9\) One of the major cytokines produced by VSMC is MCP-1, a chemokine that is responsible for leukocytes recruitment to the vessel wall in the early events of atherosclerosis,\(^10\) playing an important role in the migration, activation of monocytes and T cells and also regulating the proliferation of VSMC.\(^11\,^12\)

Besides MCP-1 can act also as a great potential mitogenic marker for VSMC.\(^13\) These cells respond to mitogen stimuli such as proliferation, migration into the intima and the secretion of matrix products such as collagen, fibronectin, elastin and proteoglycans.\(^10\,^13\,^14\)

NF-\(\kappa\)B is a transcription factor that regulates genes involved in the inflammatory response, apoptosis, cell proliferation and increased production of reactive oxygen species (ROS), contributing expressively to activation of several pathways and MCP-1 expression.\(^15\) This transcription factor is mainly involved in stress response, immunity and inflammation, and can be activated by cells exposure to LPS, free radicals, viral infection, ultraviolet (UV), B or T cells, and also by inflammatory cytokines such as MCP-1.\(^4\) There are several inhibitors of NF-\(\kappa\)B, including JSH-23 (4-Methyl-N1-(3-phenylpropyl) benzene-1,2-diamine), which acts as a selective blocker of nuclear translocation of p65.\(^16\)

Studies conducted by our group had shown that with the development of CKD and consequent accumulation of uremic toxins, there is an activation of inflammatory response (mediated by pro-inflammatory cytokines) and especially vascular inflammatory response (mediated by chemokines and adhesion molecules).\(^17\) Therefore, the interaction between the intimal and medium vessel layers with PC and PCS could hypothetically, via NF-\(\kappa\)B transcription factor, reflect in elevated plasma levels of vascular inflammation markers such as MCP-1. Thus, to clarify the mechanisms involved in the development of uremic cardiovascular disease (CVD), we investigated the *in vitro* role of PC and PCS in MCP-1 expression via transcription factor NF-\(\kappa\)B p65 in VSMC.

**METHODS**

**SYNTHESIS AND CHARACTERIZATION OF \( p \)-CRESYL SULFATE (PCS)**

PC was purchased from Sigma-Aldrich Co. (StLouis, MO, USA). PCS was synthesized as described by Feigenbaum and Neuberger\(^18\) using chlorosulfonic acid, resulting in the potassium salt of the compound (Figure 1). PC and PCS stock solutions at 5000 mg/L were prepared by diluting them directly with methanol, and aliquots were stored at minus 20°C (1 mL). The final concentrations of both toxins (47.20 to 0.60 mg/L) were obtained by defrosting and diluting the stock solution in PBS immediately prior to the experiments.
Figure 1. Synthesis of p-cresyl sulfate salt from p-cresol

HPLC FINGERPRINT AND NMR ANALYSIS

The spectra were obtained in the HPLC chromatograph ProStar Gradient Varian, bomb ProStar 230, photodiode array detector 335, loop 50 µL. It was used LiChrosorb RP-18 column as the stationary phase, and 50 mM of ammonium formiate and methanol HPLC grade as the mobile phase, with a concentration gradient of 65-25% A (0-15 min), 25-65% B (15-20 min), with balance between time and gradients concentrations of 2 min. The flow rate was 1.0 mL/min and wavelength of excitation/emission 214/306 nm. NMR analysis (1H and 13C) were carried out using a Bruker Avance III 400 MHz spectrometer. The samples (50 mg) were dissolved in D2O and the 1H and 13C chemical shifts were expressed in ppm (d) relative to TMSP-d4 (2,2,3,3-tetradeuterium-3-trimethylsilylpropionate; d = 0 for 13C and 1H).

UREMIC TOXINS PREPARATION

For the concentrations used in our experiments, we referred to the list of uremic toxins provided by the European Uremic Toxin Work Group (EuTox- http://eutoxdb.odeesoft.com/index.php). Thus, we studied PC and PCS at normal (0.60 mg/L and 2.87 mg/L), uremic (15.60 mg/L and 20.10 mg/L) and maximum uremic concentrations (40.20 mg/L and 47.70 mg/L).

VASCULAR SMOOTH MUSCLE CELLS ISOLATION, CULTURE AND CHARACTERIZATION

All experiments were conducted in accordance with the Health Ethics Committee from the Universidade Federal do Paraná (number 1140.065.11.06, CEP/SD) and a consent form for umbilical cord utilization was obtained from the mothers. Human vascular smooth muscle cells (VSMC) were isolated as previously described by Martín de Llano et al.19 Basically the umbilical vein was cannulated followed by subsequent enzymatic digestion with collagenase (C6885, Sigma, St. Louis, USA). Subsequently, VSMC were cultured with 1% gelatin (Sigma, St. Louis, USA) coated flasks, with DMEM-199 (Gibco, Carlsbad, USA) supplemented with 15% of fetal bovine serum (Gibco, Carlsbad, USA), 100 U/mL of penicillin and 50 mg/mL of streptomycin (Gibco, Carlsbad, USA), maintained in culture flasks and incubated at 37°C and 5% of CO2. VSMC were then characterized by staining with monoclonal anti-α-actin antibody (Dako Cytomation, clone JC70A), and also by their morphological characteristics evaluated by optical microscopy (Nikon, Melville, USA). For all the experiments, cells of the third and fourth passage were used.

CELL VIABILITY ASSAY

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previous described.20 Briefly, VSMC cells (10^4 cells/well) were plated in 96 well plates. After 24 hours incubation, the media was changed and the cells were treated with PC and PCS. Then, the media was replaced with fresh media (100 µL/well) and 10 µL of MTT (Sigma, St. Louis, USA) solution (5 mg/mL in D-PBS) was added to each well. The plate was left for further 4 hours in the incubator at 37°C. Subsequently, the media was removed and replaced by dimethylsulfoxide (DMSO) (Sigma, St. Louis, USA) to dissolve the crystals of reduced Formazan. The absorbance was measured at 570 nm (Tecan, Männedorf, Switzerland).

VSMC TREATMENT WITH PC AND PCS

VSMC were plated (10^4 cells/well) in 96 wells plates, coated with gelatin 1% and incubated at 37°C and 5% CO2 for 8 hours with DMEM-199 supplemented. Then, underwent a 12 hour period of starvation with DMEM-199 and 0.3% FBS. For treatment, PC e PCS were diluted in Krebs-Ringer Phosphate Buffer (KRP) pH 7.4, at normal, uremic and maximal uremic concentrations of PC and PCS with or without 30 µM of NF-κB p65 pathway inhibitor JSH-23 (Sigma, St. Louis, USA) for 3 hours.21-23 VSMC were also incubated alone with KRP. Finally, the supernatants were collected and stored at minus 20°C for subsequent quantification of MCP-1.

MCP-1 SUPERNATANT LEVELS

MCP-1 was measured by an enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies (R&D Systems, Minneapolis, USA).
The concentrations (pg/mL) were calculated by reference to standard curves performed with the corresponding recombinant molecule. The ELISA system measuring range was 31.25 - 2000 pg/mL. The intra-assay and inter-assay coefficient of variation (CV) was 6.0 and 6.2% respectively. The protocol and concentrations used and the concentrations, followed the manufacturer’s recommendations. The absorbance was read at 450 nm with a reference filter at 570 nm in a microplate reader (Tecan, Männedorf, Switzerland). It was carried out five experiments in triplicate.

**DATA ANALYSIS**

Statistical analyses were conducted using the statistical packages JMP version 8.0 (SAS Institute Inc., Cary, N.C., USA) and SigmaStat version 3.5 (Systat software Inc., Erkrath, Germany). Determination of significant differences was performed using Student t test or ANOVA for paired data and Mann-Whitney and ANOVA on Rank’s for unpaired data. Values were expressed as mean ± standard mean error (SME) of the five experiments in triplicate. Results were considered significant when $p < 0.05$.

**RESULTS**

**p-cresyl Sulfate Characterization**

HPLC analysis has shown the presence of mainly 4 compounds at retention times of 4.23 (14.05%), 7.08 (6.90%), 8.19 (74.00%) and 13.54 (5.05%). The peak at 8.19 min was found to be for p-cresyl sulfate (Figure 2A). For the same chromatographic conditions used, p-cresol compound had a retention time of 13.99 min (98.08%) (Figure 2B).

**Spectroscopic Data of p-cresyl Sulfate**

$^1$H NMR (D$_2$O, 400 MHz): $d$ 2.31 (s, 3H, H$_3$), 7.18 (d, 2H, $J$ 8.80, H$_2$-6), 7.24 (d, 2H, $J$ 8.40, H$_3$-5),$^{13}$C NMR (D$_2$O, 400 MHz): $d$ 20.0 (C$_9$), 121.41 (C$_{2,4}$), 130.24 (C$_{3,3'}$), 136.5 (C$_8$), 148.99 (C$_9$).

**VSMC Culture and Characterization**

The cultured cells were visualized by light microscopy (Optiphase, Van Nuys, CA), showing the following morphological characteristics: long, spindle-shaped cells with no transverse grooves, with single nucleus, suggesting VSMC. After extraction and cultivation, the cells were characterized by detection of $\alpha$-actin filament, cytoskeletal protein characteristic of smooth muscle cells, responsible for contraction and migration. For this characterization, $\alpha$-actin (Sigma-Aldrich, St. Louis, USA) monoclonal primary antibody and the secondary antibody anti FITC (Sigma-Aldrich, St. Louis, USA) was used. Cells were also stained with DAPI-4',6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, USA) in order to locate the core and consequently the presence of cells. The cultured cells were visualized by fluorescence microscopy (Nikon Instruments Inc.).

**Cell Viability Assay**

VSMC viability was assessed by MTT. VSMC were cultured with PC and PCS at normal, uremic and maximum uremic concentrations. All the concentrations of both toxins tested had no significant effect on VSMC viability.

**Effect of PC and PCS on MCP-1 Expression and NF-κB p65 Pathway Blockage**

Data in Figure 3 show that VSMC treated with PC at normal (PCn), uremic (PCu), maximal uremic (PCm). PCu and PCm found a significant MCP-1 increase after 3 hours (137.10 ± 18 pg/mL and 155.5 ± 15.0 pg/mL, $p < 0.001$) when compared to control (34.0 ± 3.5 pg/mL). When VSMC were treated with NF-κB p65 inhibitor, there was a significant decrease in MCP-1 production after 3 hours of treatment with PCu and PCm ($p < 0.005$) (137.1 ± 18.3 vs. 36.4 ± 10.5 and 155.5 ± 15.0 vs. 63.4 ± 15.5 pg/mL respectively).

For all PCS concentrations tested, normal (PCSn), uremic (PCSu) and maximal uremic (PCSm), the MCP-1 production was similar (156.5 ± 19.5; 155.6 ± 4.2 and 151.2 ± 14.2 pg/mL respectively) as shown in Figure 4. Interestingly NF-κB p65 pathway inhibitor had no significant effect on MCP-1 production after PCS treatment.

It was also observed (Figure 5) that the expression of MCP-1 by VSMC treated with PCm and PCSm were similar after 3h (155.5 ± 15.0 and 151.2 ± 14.2 pg/mL, respectively). In addition, in the presence of NF-κB p65 inhibitor, there was a significant decrease in MCP-1 production after 3 hours of treatment with PCu and PCm ($p < 0.005$) (137.1 ± 18.3 vs. 36.4 ± 10.5 and 155.5 ± 15.0 vs. 63.4 ± 15.5 pg/mL respectively) (Figure 3). This effect was not observed for PCS (Figure 4).
Figure 2. HPLC fingerprint of the p-cresyl sulfate (2A) and p-cresol (2B).

Figure 3. Effect of PC on MCP-1 production in VSMC. VSMC were incubated with PC at normal (PCn), uremic (PCu) and maximal uremic (PCm) concentrations with or without NF-κB p65 pathway inhibitor (i) for 3 hours. Data are expressed as mean ± SEM of five independent experiments in triplicate. * p < 0.001 - Control vs. PCu and PCm; ** p < 0.005 - PCu vs. PCui and PCm vs. PCmi.

Figure 4. Effect of PCS on MCP-1 production in VSMC. VSMC were incubated with PCS at normal (PCSn), uremic (PCSu) and maximal uremic (PCSm) concentrations with or without NF-κB p65 pathway inhibitor (i) for 3 hours. Data are expressed as mean ± SEM of five independent experiments in triplicate. * p < 0.001 - Control vs PCSn, PCSu, and PCSm.

Figure 5. Effect of maximal uremic concentrations (PCm and PCSm) on MCP-1 production in VSMC. VSMC were incubated with PC and PCS at maximal uremic concentration. Data are expressed as mean ± SEM of five independent experiments in triplicate. * p < 0.001 - Control vs PCm and PCSm.

**Discussion**

PC and PCS are well-known uremic toxins that are first uptaken by the kidneys, blood vessels, bones, and across the blood-brain barrier via organic anion transporters (OATs). Both toxins induce the production of several oxygen free radicals and inflammatory molecules, such as the chemokine MCP-1, important in the early events of uremic cardiovascular process. We recently demonstrated that MCP-1 is elevated in CKD, and its plasmatic concentrations are inversely correlated to the glomerular filtration rate (GFR) and CKD stage. In addition when endothelial cells are in contact with uremic serum, there are an increase in MCP-1 production in a time and uremic concentration way. The main finding of the present study is that PC and PCS were capable of inducing MCP-1 production in human VSMC. This expression occurs in a dose dependent way and mainly via NF-κB.
p65, after PC exposure. On the other hand, PCS even in the normal concentration was able to stimulate MCP-1 production, which seems to occur by a different pathway, since the production of MCP-1 was not reduced after VSMC treatment with a NF-κB p65 inhibitor.

In CKD, the initiation of the atherosclerosis process takes place by endothelium aggression by uremic toxins, which triggers a process of systemic inflammation involving numerous inflammatory cells that become activated and release a variety of molecules, such as MCP-1, one of the most important molecules in atherosclerosis pathophysiology. As endothelial cells, VSMC can express a variety of cytokines and adhesion molecules and thus contribute to the initiation and propagation of inflammatory response in the atherogenic process to attract and activate leukocytes, inducing cell proliferation, endothelial dysfunction, thereby promoting and stimulating the production of extracellular matrix components.

MCP-1 is a chemokine produced by a variety of cell types either constitutively or after induction by oxidative stress, cytokines or growth factor and has been linked with chronic inflammatory diseases, antitumor immunity, atherosclerosis and cervical cancer. In CKD patients it was found that serum levels of MCP-1 were significantly higher than control subjects independently of association with CVD. In vitro it was described that PC stimulates the production of MCP-1 in VSMC also treated with pro-atherogenic lipoproteins.

In addition, Chang et al. found that PC can potentially prevent the formation of blood clots, causing bleeding disorders through inhibition of platelet aggregation. Meijers et al. suggested that PC levels measured in CKD patient’s plasma can be useful to predict the cardiovascular risk, and it can be useful as a traditional glomerular filtration marker. PCS has been described as an effector of pro-inflammatory leukocytes and consequent increase in free radicals production. This effect may contribute to the development of CVD in uremic population.

Also, PCS was correlated with endothelial function imbalance in patients who are undergoing dialysis therapy. Furthermore high levels of PCS are associated with the pathogenesis of CVD, particularly atherosclerosis in patients with kidney failure. Meijers et al. suggested that PCS and indoxyl sulfate (another important protein bound uremic toxin) can be valid to monitor the behavior of protein-bound solutes during dialysis. However, both are inhibitors of competitive binding to the same binding site of albumin, so the technique can be useful, but not specific.

The present study is the first to demonstrate the in vitro effect of PC and PCS in MCP-1 production in human VSMC. Our findings suggest that this expression occurs by NF-κB p65 pathway to PC but by a different NF-κB subunit, once NF-κB p65 blockage was not effective in decreasing the MCP-1 production after VSMC stimulation with PCS. One of the main routes of activation of MCP-1 production pathway is NF-κB whose modulation triggers a cascade of signaling events, which control gene expression. It was already demonstrated that PCS and indoxyl sulfate, regulate the expression of MCP-1 and adhesion molecules by activation of reactive oxygen species (ROS) through NF-κB activation in vascular endothelial cells. The transcription factor NF-κB p65/p53 is a heterodimer originally identified as a nuclear factor, reported to be involved in the expression of various genes in different types of cells, and the subunit p65 is closely related to promote the infiltration of monocytes. In addition NF-κB p65 subunit are directly involved in the regulation of MCP-1 expression. Dwarakanath et al. described in human VSMC that the NF-κB p65 pathway in directly involved in the regulation of several genes and inhibition of this pathway could result in a reduction of MCP-1 expression.

The NF-κB pathway has been implicated in almost all chronic diseases, and several publications discuss the selective blockage of this pathway as a therapeutic strategy using different cell models. In immune diseases such as rheumatoid arthritis selective blockage of NF-κB could control the extravasation of inflammatory cells. In bone cells, selective inhibition of NF-κB activation markedly decreases pro-inflammatory cytokine production and bone loss. In dendritic cells selective NF-κB inhibition may provide a tool to decrease cellular immune responses after transplantation. In endothelial cells, NF-κB inhibitions impair the ability of angiostatic agents to sprouting of endothelial cells and to overcome endothelial cell anergy. This is of special interest because, in tumor cells, NF-κB activation has been associated to inhibition of apoptosis.
Finally, agents that can inhibit protein kinases, protein phosphatases, proteasomes, ubiquitination, acetylation, methylation, and DNA binding steps have been identified as NF-κB inhibitors, aimed treating different diseases.\(^\text{36}\) In addition to NF-κB, other transcription factors such as PPAR (peroxisome-proliferator-activated receptors) and LXR (Liver X receptors) which regulate the expression of genes that control lipid and lipoprotein metabolism and glucose homeostasis also act in the predisposition to atherosclerosis and inflammation.\(^\text{37}\) Also, it was described that MCP-1 induces activation of MAP-kinases ERK, JNK and p38 MAPK in human endothelial cells\(^\text{38}\) and JAK/STAT pathway induced activation of cytokines in vascular smooth muscle cells.\(^\text{39}\)

A recent publication by Vanholder \textit{et al.}\(^\text{40}\) argue about the value of studies with PC since in the CKD patients circulation the concentration of PC is minimum, being rapidly converted to its conjugate PCS. Although they reinforce the importance of studies with PC, in order to compare PC with PCS and analyze the \textit{in vitro} effects of these two important toxins. In fact we observed in this study that PCS is more aggressive to VSMC even in the lowest concentration used, which confirm that PCS is the effective toxin. Indeed, there is a lot of speculation if the addition of a sulfate group in the PC molecule is the responsible for the harmful effects of this toxin to the body, and until now, there are few \textit{in vitro} studies elucidating the mechanisms behind it. When in contact with VSMC, sulfate group alone and even BSA alone (at 4%) were not able to induce MCP-1 expression (data not show) what makes us think how PCS interacts with the cells. For us this and others remarks in PCS cell uptake remains unclear and more studies are needed to further clarify how PCS interacts with a range of cells, including VSMC.

We recognize that this study has some limitations and that more work will be needed. First, there are other activation pathways and NF-κB subunits that are involved in the expression of MCP-1. Furthermore, it is important to use other methods to investigate the MCP-1 expression after VSMC stimulation with PC and PCS. Additionally, the receptor expression analysis to MCP-1 (CCR2) in VSMC can be of great importance in understanding these cellular mechanisms.

**Conclusion**

In conclusion, this study shows that VSMC are involved in atherosclerosis lesion formation and cytokines production such as MCP-1, which contributes to the inflammatory response initiation and propagation to lipids. Our results suggest for the first time that PC mediates MCP-1 production in VSMC, probably through the NF-κB p65 pathway, although we suggest that PCS acts through a different subunit pathway since NF-κB p65 inhibitor was not able to inhibit MCP-1 production. We believe that strategies to block the pathways enrolled in MCP-1 expression can be useful as targets in the early events of atherosclerosis and in this way reduce the harmful effects of uremic toxicity in CKD patients.

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