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The immunomodulatory effect of Poria cocos polysaccharides is mediated by the Ca^{2+}/PKC/p38/NF-κB signaling pathway in macrophages

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ABSTRACT

Poria cocos polysaccharide (PCP), extracted from Poria cocos sclerotium, has many biological activities. The present study explored the immunomodulatory effect and the underlying molecular mechanism of PCP in RAW 264.7 macrophages. Griess reaction, ELISA assays and confocal laser scanning microscopy revealed that the production of nitric oxide (NO), TNF-α, IL-1β, IL-6 and intracellular calcium level were increased by PCP. However, this effect on cytokines was suppressed with a Ca^{2+} channel blocker or a p38 inhibitor, which indicates that Ca^{2+} and p38 are crucial to the immunomodulatory effect of PCP. We further demonstrated that PCP-treated cells also exhibited increased the activity of PKC, mRNA and protein expression levels of p38 and NF-κB, which is also reduced with a Ca^{2+} channel blocker. Taken together, the Ca^{2+}/PKC/p38/NF-κB signaling pathway may involve in the immunomodulatory effects of PCP.

1. Introduction

Poria cocos is a common traditional Chinese medicine with diuretic, sedative [1], and immunomodulatory effects [2]. Poria cocos polysaccharide (PCP), extracted from Poria cocos sclerotium, has been reported to be beneficial to human health [3]. Recent studies have shown that PCP exhibits antitumor [1], hepatoprotective [4], mitogenic [5], and also immunomodulatory effects [6]. It's relative molecular weight is about 160 kDa [7]. PCP was composed of ribose, arabinose, xylose, mannose, glucose, and galactose in the molar contents of 1.49, 1.17, 0.62, 10.34, 86.39 and 1.31 μM, respectively [8]. However, the molecular mechanisms of immunomodulatory effects of PCP have not yet been elucidated clearly.

Previous studies have shown that PCP is involved in immune regulation by activating NF-κB through the CD14, TLR4, and CR3 membrane receptors in macrophages [9]. However, there are few studies regarding PCP can play an immunomodulatory effect by increasing Ca^{2+}.

Ca^{2+}, as a multifunctional cation, acts as a second messenger in the immune cell including macrophages, T and B lymphocytes. The amount and duration of the flow of Ca^{2+} will affect the type and duration of intracellular signal transduction [10]. Further calcium signaling is central to many cellular processes including immune responses [11], which can involve in cytokines production by activating NF-κB [12].

Protein kinase C (PKC), as a downstream molecule of Ca^{2+}-dependent signaling pathways [13], is a ubiquitous serotonin protein kinase that catalyzes the phosphorylation of many cell proteins. It can associates with receptors on the surface of immune cells to activate downstream signal transduction cascades and play an important role in immune responses [14].

Lipopolysaccharides (LPS) are bacterial components that can stimulate the secretion of proinflammatory cytokines, including TNF-α and IL-6 in macrophages through the TLR4/NF-κB signaling pathway [15]. Furthermore, many reports have shown that Ca^{2+} plays an important role in LPS-induced NF-κB activation [16,17] by stimulating the activity of downstream Ca^{2+}-dependent signaling pathways, such as PKC [18].

In the present study, we investigated the immunomodulatory effect of PCP and the ability of PCP to activate NF-κB via the Ca^{2+}/PKC/p38 signaling pathway in RAW264.7 macrophage cells. LPS was used as a positive control to stimulate RAW264.7 macrophage cells and $NiCl_2$ was used, as it is a calcium channel blocker that can reduce the intracellular Ca^{2+} concentration [19–21]. And SB203580 was as a p38 inhibitor.

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2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), 0.25% EDTA/trypsin, and fetal bovine serum (FBS) were obtained from Hyclone (Logan City, UT, USA). LPS (extracted from *Escherichia coli* O111: B4) and the Ca^{2+} channel blocker, $NiCl_2$, were purchased from Sigma-Aldrich (MO, USA). The p38 inhibitor SB203580 was purchased from MedChem Express (NJ, USA). Ci Yuan Biotechnology Co., Ltd. (Xian, China) was the source of PCP (> 90% pure). The endotoxin level in PCP was < 0.1 EU/μg detected by Tachypleus amebocyte lysate (TAL) assay using Bioendo™ EC Endotoxin Test Kit (Xiamen Bioendo Technology Co., Ltd., Xiamen, China). Griess reagent Kit was from Beyotime Biotechnology (Shanghai, China).

2.2. Cell culture

The murine macrophage cell line RAW 264.7 was a gift from Professor Jianping Gong (Department of Hepatobiliary Surgery, the Second Affiliated Hospital of Chongqing Medical University). The cells were maintained in DMEM (supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells treated with LPS were considered as positive controls; while cells cultured in DMEM without any other treatment were considered as controls. Cells were treated with or without LPS (100 ng/ml), PCP (200 μg/ml) or PCP (200 μg/ml) together with $NiCl_2$ (1 mM) or PCP (200 μg/ml) together with SB203580 (20 μM).

2.3. Griess reaction

Macrophages (5×10^4 cells/ml) were seeded onto 24-well culture plates and treated with or without LPS (100 ng/ml) or PCP (200 μg/ml) for different periods of time (4 h, 8 h, 16 h, 24 h, 32 h, 48 h and 72 h). NO levels of isolated culture medium in different time groups were determined by the Griess reaction. Then, the isolated culture media were incubated with an equal amount of Griess solution (1% sulfanilamide, 0.1% naphthylethydiamine dihydrochloride, and 2% phosphoric acid). The accumulation of NO^{2-}, an indicator of NO production, was determined with a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA) at 540 nm with a sodium nitrite standard curve.

2.4. ELISA

Macrophages (5×10^5 cells/ml) were seeded onto 24-well culture plates treated with or without LPS (100 ng/ml), PCP (200 μg/ml), or PCP (200 μg/ml) and $NiCl_2$ (1 mM) or PCP (200 μg/ml) and SB203580. The culture supernatant was collected from each group, and the levels of TNF-α, IL-1β, and IL-6 were analyzed using the corresponding commercial mouse ELISA kits (4A Biotech, Beijing, China) according to the manufacturer's instructions. The absorbance was measured at 450 nm with a microplate reader (Mul-tiskan FC, Thermo, Waltham, MA, USA). A standard curve prepared from standard-samples TNF-α, IL-1β, and IL-6 was used to calculate cytokines production.

2.5. Measurement of PKC activity

Macrophages (10^7 cells/well) were seeded onto 6-cm dishes and incubated in DMEM with treatments as mentioned above for different periods of time (4 h, 8 h, 16 h, 24 h, and 48 h). Cells were collected and PKC levels were measured using the PKC activity quantitative detection kit (Genmed Scientific Inc., Shanghai, China). Total protein was extracted and protein concentration was measured using BCA protein assay kit (Beyotime, Nantong, China). Then equal amounts of protein added with the appropriate reagent in turn according to the instructions. Finally, absorbance was measured at 340 nm with a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

2.6. Measurement of intracellular calcium concentration

Macrophages (10^3 cells/well) were seeded onto the confocal plate and incubated for 24 h. The cells were washed with Hanks' Balanced Salt Solution (HBSS) and then incubated in DMEM containing 8 μM Fluo-4/AM (Dojindo, Mashiki, Japan) and 0.04% Pluronic acid F-127 for probes to get better into cells at 37 °C for 50 min. Next, cells were washed 3 times with HBSS to remove the remaining Fluo-4/AM and Pluronic acid F-127, then treated with PCP or PCP and $NiCl_2$, as mentioned above. And then the cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. To visualize nuclei, cells were stained with 10 μg/ml DAPI. The confocal laser scanning microscope (Nikon, Tokyo, Japan) was used to detect the fluorescent images of the intracellular calcium value.

2.7. qRT-PCR

The total mRNA in cells was extracted with TRIzol reagent and reverse transcribed into cDNA with the Prime Script RT-PCR kit (Takara, Dalian, China). qRT-PCR was performed with the CFX-96 Touch™ real-time PCR system (Bio-Rad, Foster City, CA, USA) using the SYBR-Green method and began with initial denaturation for 30 s at 95 °C, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 58 °C for 30 s. The relative mRNA expressions of p38 and NF-κB were calculated by the comparative Ct method and normalized to the endogenous reference β-actin. Reactions were run in triplicate and the following qRT-PCR primer pairs were used: p38 (forward: 5′-GTACCA CGATCCTGATGATG-3′ and reverse: 5′-CAGTAGAGTGGGATCAA CAG-3′), NF-kB (forward: 5′-CCAAAGAAGGACACGACAGAATC-3′ and reverse: 5′-GGCAGGCTATTGCTCATCACA-3′), and β-actin (forward: 5′-AGATTACTGCTCTGGCTCCTAGC-3′ and reverse: 5′-ACTCATCGTAC TCCTGCTTGCT-3′).

2.8. Western blot

The proteins were extracted from the treated macrophage cells with the Total Protein Extraction Kit (Keygen, Nanjing, China). Then the protein concentration in the lysates was evaluated using a BCA protein assay kit (Beyotime, Nantong, China). After denaturation, equal amounts of protein were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Boston, USA). Then the membranes were blocked with 5% (w/v) skimmed milk and incubated with rabbit anti-p-p38 antibody (1:800, Cell Signaling Technology, USA), rabbit anti-p-NF-kB antibody (1:1000, Cell Signaling Technology, USA), and mouse anti-β-actin antibody (1:1000, Beyotime, China) at 4 °C overnight. After washing three times for 30 min, the membrane was incubated with HRP-conjugated secondary antibodies (1:1000, Beyotime, China). Finally, the blots were visualized using an enhanced chemiluminescence (ECL) kit (Millipore, Boston, USA).

2.9. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and analyzed using SPSS 20.0 (IBM, Armonk, NY, USA). Group comparison was performed using one-way analysis of variance (ANOVA) and *t*-test. Experiments were performed in triplicate. $p < 0.05$ was considered statistically significant.

3. Results

3.1. PCP induces nitric oxide (NO) production in macrophages

To determine the effects of PCP on NO production, we detected the accumulation of nitrite using Griess reagent. As shown in Fig. 1, PCP

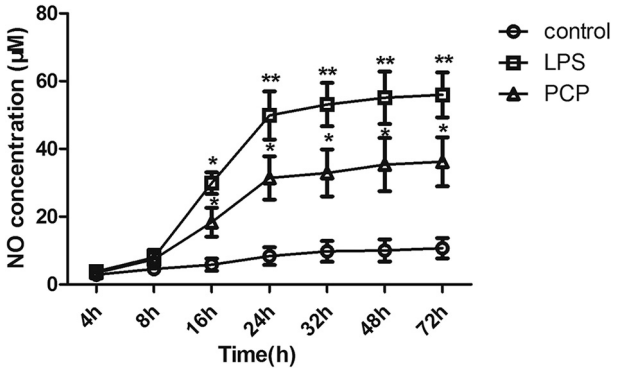

Fig. 1. Effects of PCP on NO production by RAW 264.7 macrophage at different time points (4 h, 8 h, 16 h, 24 h, 32 h, 48 h and 72 h). After macrophages were cultured with PCP (200 μg/ml) or LPS (100 ng/ml), the NO concentration was detected by Griess reaction using the culture medium. * $p < 0.05$, ** $p < 0.01$ vs. control group.

and LPS significantly increased ($p < 0.05$) NO production compared with controls from 16 h–72 h. This demonstrates that PCP can stimulate NO production in macrophages.

3.2. PCP enhances [Ca^{2+}]i in macrophages

Since Ca^{2+} plays an important role in NF-κB activation and the cytokines secretion, including TNF-α and IL-6 [20,22], we have a test for analyzing the effects of PCP on [Ca^{2+}]i by measuring fluorescence intensity. The cells were exposed to PCP, with or without pretreatment with $NiCl_2$. As shown in Fig. 2, the fluorescence intensity of PCP-treatment cells significantly increased (2.39-fold, $p < 0.05$) compared with controls. While cells treated with PCP and $NiCl_2$ exhibited significantly decreased fluorescence (0.46-fold, $p < 0.05$) compared with PCP treatment alone. These findings demonstrate that Ca^{2+} may be a necessary factor for PCP to perform immune effects.

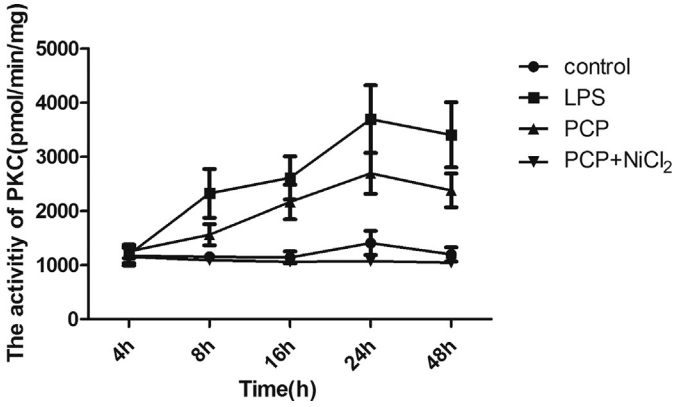

Fig. 3. The PKC activity at different time points in RAW 264.7 cells treated with or without PCP ((200 μg/ml), PCP (200 μg/ml) and $NiCl_2$ (1 mM). The level of PKC activity was measured by power-spectral method. The PKC activity was significantly induced by PCP from 8 h–48 h, but this effect was reduced with $NiCl_2$.

3.3. PCP stimulates PKC activity in macrophages

PKC, as a Ca^{2+}- and phospholipid-dependent protein kinase [23], has an important effect on the activation of NF-κB [13]. To further exclude the possibility that PKC is downstream of cell immunomodulatory pathway, we determined PKC activities. As shown in Fig. 3, PCP/LPS treatment significantly increased ($p < 0.05$) PKC activity in macrophages from 8 h–48 h. However, PKC activity was significantly decreased ($p < 0.05$) in cells treated with the presence of $NiCl_2$. These results suggested that there may be a correlation between PKC and immunomodulatory effect of PCP.

3.4. PCP affects p38 and NF-κB mRNA and protein expression

In order to further study the immunoregulatory mechanism of PCP, we measured the mRNA and protein expression levels of p38 and NF-κB with qRT-PCR (Fig. 4A) and Western blot (Fig. 4B, C). PCP and LPS

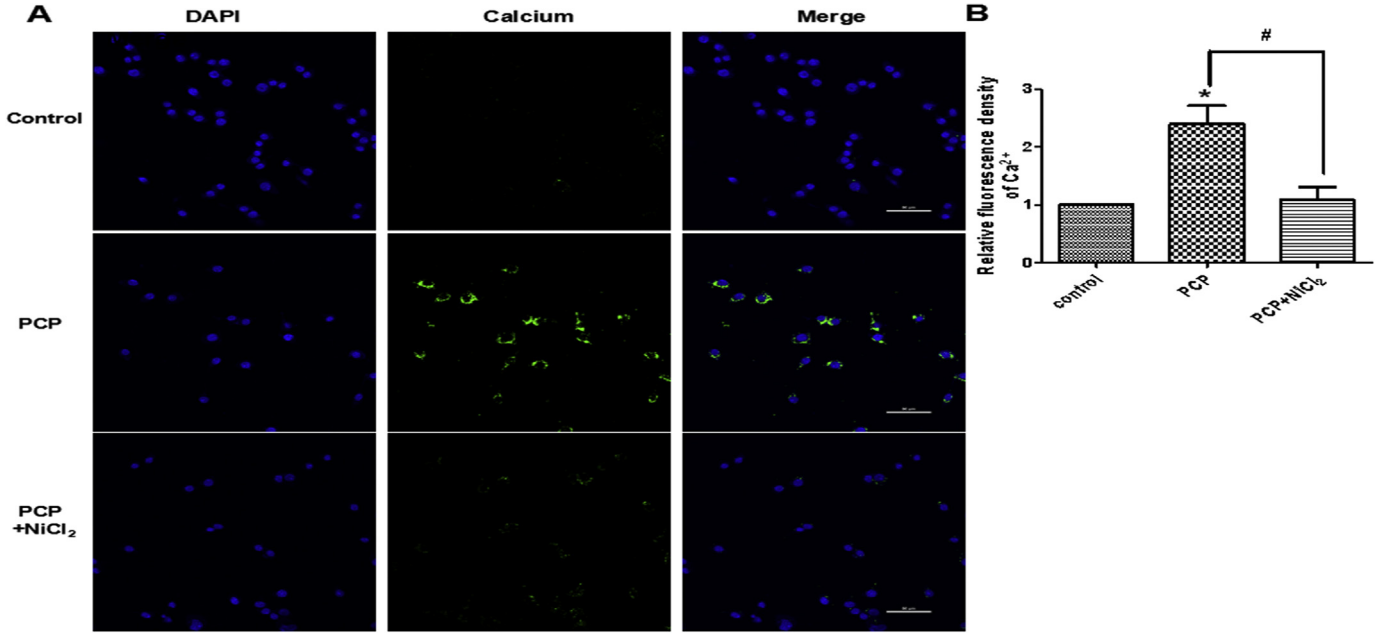

Fig. 2. The magnitude of [Ca^{2+}]i in RAW 264.7 macrophage cells was detected by confocal laser scanning microscope. Fluorescence imaging in RAW 264.7 cells is demonstrated. (A) RAW 264.7 cells were treated with or without PCP or PCP and $NiCl_2$. Cells were stained with calcium (green) and DAPI (blue). (B) Relative fluorescence intensity of Ca^{2+} Data was represented as mean ± SD. * $p < 0.05$ vs. control, # $p < 0.05$ vs. PCP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

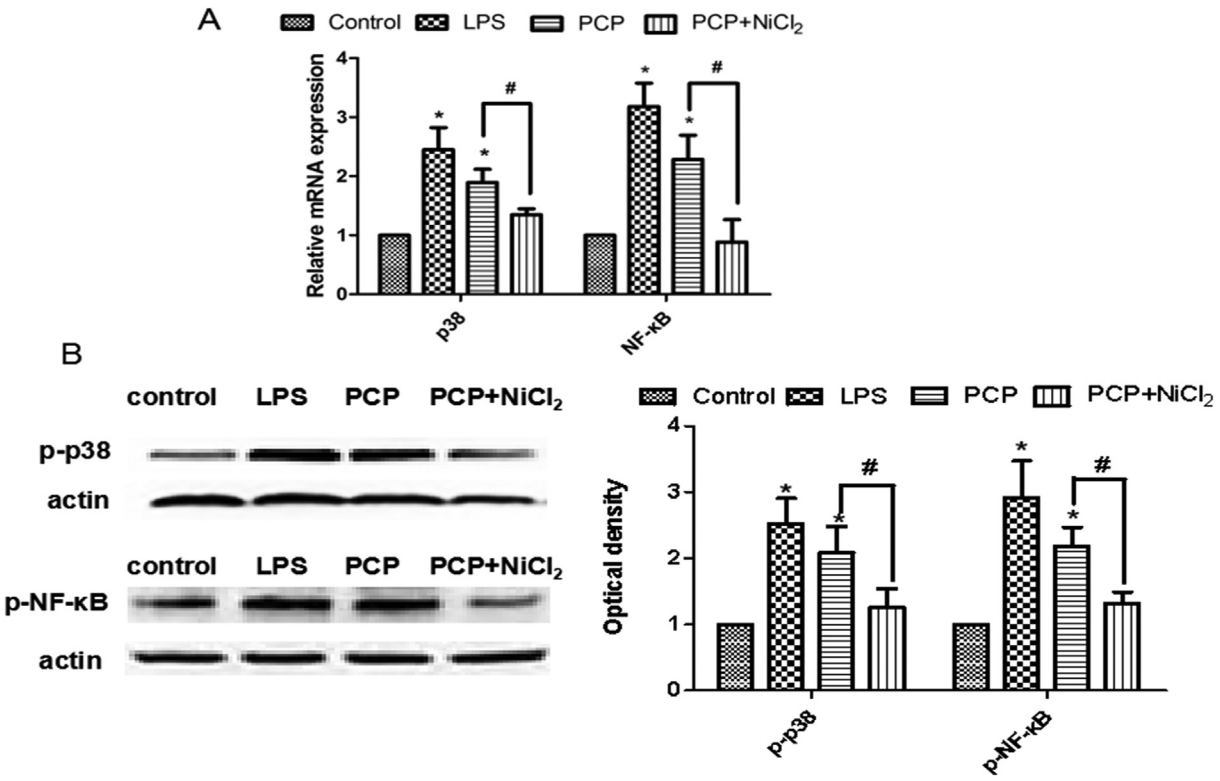

Fig. 4. The relative expressions of mRNAs and proteins of p38 and NF-κB in macrophages. (A) The mRNA expression was detected with qRT-PCR. (B) The protein expression was detected with Western blot. * $p < 0.05$ vs. control, # $p < 0.05$ vs. PCP.

significantly increased the mRNA and protein expression of p38 and NF-κB compared with controls ($p < 0.05$). However, the expression levels of p38 and NF-κB were significantly reduced in cells treated with PCP and $NiCl_2$ compared with PCP-treated cells ($p < 0.05$). These results suggest that p38 and NF-κB may be also involved in the immunoregulatory effect of PCP.

3.5. PCP affects TNF-α, IL-1β, and IL-6 expression levels

To investigate the immunomodulatory effects of PCP on macrophages, we examined the levels of cytokines by ELISA. The levels of TNF-α, IL-1β, and IL-6 were significantly elevated after PCP or LPS treatment ($p < 0.05$) and significantly decreased ($p < 0.05$) after treatment with the presence of $NiCl_2$ or SB203580 (Fig. 5A, B, C). These results suggest that PCP can stimulate macrophages to secrete cytokines, and Ca^{2+} and p38 are crucial for TNF-α, IL-1β, and IL-6 production.

4. Discussion

PCP has been reported to have multiple immune effects, such as promoting the production of B lymphocyte antibodies and spleen antibodies [6,9], increasing levels of serum antibody IgG, and enhancing phagocytosis of peritoneal macrophages [24]. In recent years, studies have shown that many plant polysaccharides play an immune role through the TLR4/NF-κB signaling pathway [25,26] and PCP may activate NF-κB through TLR4 [9]. However, whether other pathways besides the TLR4/NF-κB signaling pathway are also involved in the immune effects of PCP remains unclear. In the present study, we determined the immunomodulatory effects and underlying molecular mechanisms of PCP in macrophages.

Many plant polysaccharides play an immunomodulatory role by activating macrophages [27-29]. Macrophages, as specific antigen-presenting cells, clean foreign antigens or release cytotoxic molecules such as NO, TNF-α, and IL-1β, which are critical for successful defense against invading pathogens [30]. In the present study, PCP and LPS increased the secretion of NO, TNF-α, IL-1β, and IL-6. These findings indicate that PCP can induce the secretion of immunomodulatory factors in macrophages.

Ca^{2+} channels are known to take part in a variety of physiological processes and have been reported to activate NF-κB in macrophages [16,17]. Although previous studies have reported that plant polysaccharides have immunostimulatory properties related to increasing $[Ca^{2+}]i$ [31,32], few studies have focused on whether Ca^{2+} plays a role in the immunomodulatory effects of PCP. Therefore, we measured the magnitude of $[Ca^{2+}]i$ in macrophages. The level of Ca^{2+} increased in PCP-treated cells, whereas it decreased in cells treated with PCP and $NiCl_2$. In addition, the secretion of TNF-α, IL-1β, and IL-6 also significantly decreased after $NiCl_2$ treatment. These results suggest that Ca^{2+} is vital in the immunomodulatory function of PCP.

PKC plays a crucial role in signal transduction of various bioactive substances. Evidence shows that PKC activation plays an essential role in inducing the secretion of TNF-α and IL-1β by activating NF-κB [13,33]. In this study, we found that PCP/LPS resulted in an up-regulation of PKC activity in macrophages. Additionally, PKC activity was down-regulated after $NiCl_2$ treatment, suggesting that PKC may be the downstream molecule of Ca^{2+} involved in the immune regulation process.

The p38 kinase plays an important role in LPS-induced signal transduction pathways leading to cytokine synthesis [34] and can regulate the activation of NF-κB [35,36]. The results showed that TNF-α, IL-1β, and IL-6 were inhibited by the addition of p38 (SB203580) inhibitors. And the present study demonstrated that p38 and NF-κB were increased after PCP/LPS treatment and decreased after $NiCl_2$ treatment. Taken together, besides the classical TLR4/NF-κB signaling pathway involved in immunomodulatory effects of PCP [9], our results suggest that the Ca^{2+}/PKC/p38/NF-κB signaling pathway may be also involved in the immunomodulatory effects of PCP. However, whether

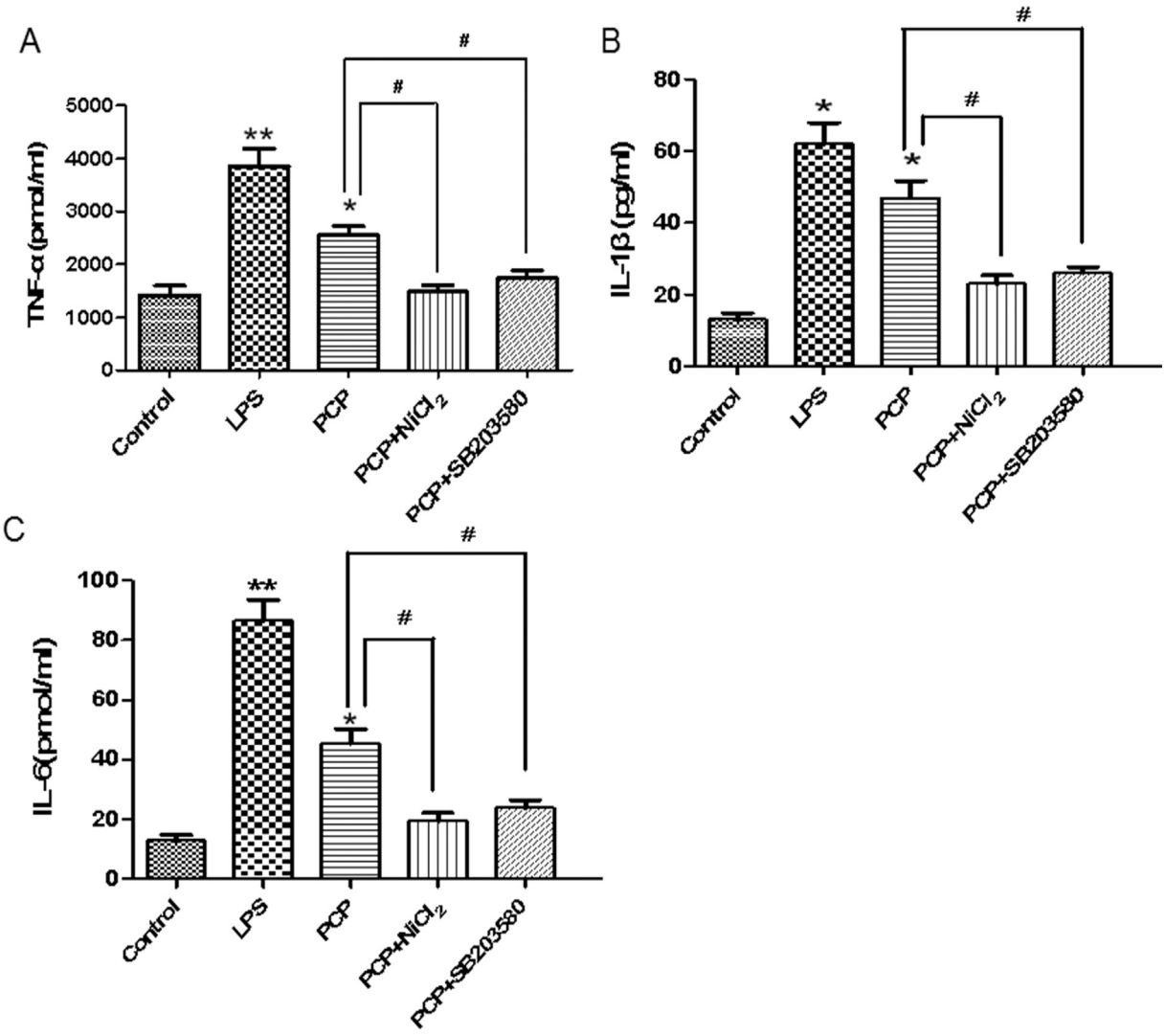

Fig. 5. The level of cytokines in RAW 264.7 cells treated with or without PCP or PCP and $NiCl_2$ or PCP and SB203580. (A) TNF-α (B) IL-1β; (C) IL-6; the secretion of cytokines were induced by PCP. However, TNF-α, IL-1β, IL-6 was suppressed with $NiCl_2$ or SB203580. * $p < 0.05$, ** $p < 0.01$ vs. Control, # $p < 0.05$ vs. PCP.

there is a link between the two pathways and whether other pathways or other receptors involved in increased intracellular calcium are unknown and need further study.

In conclusion, our studies have demonstrated an immunomodulatory effect of PCP with a molecular mechanism that is related to the activation of NF-κB through the Ca^{2+}/PKC/p38 signaling pathway.

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Conflict of interests

The authors declared no competing financial interests.

References

[1] Y. Wu, S. Li, H. Li, et al., Effect of a polysaccharide from Poria cocos on humoral response in mice immunized by H1N1 influenza and HBsAg vaccines, Int. J. Biol. Macromol. 91 (2016) 248–257.
[2] Y. Wang, M. Zhang, D. Ruan, et al., Chemical components and molecular mass of six polysaccharides isolated from the sclerotium of Poria cocos, Carbohydr. Res. 339 (2) (2004) 327–334.
[3] Y. Sun, Biological activities and potential health benefits of polysaccharides from Poria cocos and their derivatives, Int. J. Biol. Macromol. 68 (2014) 131–134.
[4] K. Wu, J. Fan, X. Huang, X. Wu, C. Guo, Hepatoprotective effects exerted by Poria Cocos polysaccharides against acetaminophen-induced liver injury in mice, Int. J. Biol. Macromol. 114 (2018) 137–142.
[5] Y. Jin, L. Zhang, L. Chen, Y. Chen, P.C. Cheung, L. Chen, Effect of culture media on the chemical and physical characteristics of polysaccharides isolated from Poria cocos mycelia, Carbohydr. Res. 338 (14) (2003) 1507–1515.
[6] X. Chen, L. Zhang, P.C. Cheung, Immunopotentiation and anti-tumor activity of carboxymethylated-sulfated beta-(1→3)-D-glucan from Poria cocos, Int. Immunopharmacol. 10 (4) (2010) 398–405.
[7] Y.Y. Chen, H.M. Chang, Antiproliferative and differentiating effects of polysaccharide fraction from fu-ling (Poria cocos) on human leukemic U937 and HL-60 cells, Food Chem. Toxicol. 42 (5) (2004) 759–769.
[8] R.D. Ke, S.F. Lin, Y. Chen, C.R. Ji, Q.G. Shu, Analysis of chemical composition of polysaccharides from Poria cocos Wolf and its anti-tumor activity by NMR spectroscopy, Carbohydr. Polym. 80 (1) (2010) 31–34.
[9] K.Y. Lee, H.J. You, H.G. Jeong, et al., Polysaccharide isolated from Poria cocos sclerotium induces NF-kappaB/Rel activation and iNOS expression through the activation of p38 kinase in murine macrophages, Int. Immunopharmacol. 4 (8) (2004) 1029–1038.
[10] J.H. Izquierdo, F. Bonilla-Abadía, C.A. Cañas, G.J. Tobón, Calcium, channels, intracellular signaling and autoimmunity, Reumatol Clin 10 (1) (2014) 43–47.
[11] R. Rooke, Can calcium signaling be harnessed for cancer immunotherapy, Biochim. Biophys. Acta 1843 (10) (2014) 2334–2340.
[12] Z. Wang, Z. Liu, L. Zhou, T. Long, X. Zhou, Y. Bao, Immunomodulatory effect of APS and PSP is mediated by Ca2+-cAMP and TLR4/NF-κB signaling pathway in macrophage, Int. J. Biol. Macromol. 94 (Pt A) (2017) 283–289.
[13] J. Yang, C. Wang, X. Nie, et al., Perfluorooctane sulfonate mediates microglial activation and secretion of TNF-α through Ca^{2+}-dependent PKC-NF-κB signaling, Int. Immunopharmacol. 28 (1) (2015) 52–60.

[14] A. Altman, K.F. Kong, Protein kinase C enzymes in the hematopoietic and immune systems, Annu. Rev. Immunol. 34 (2016) 511–538.
[15] W. Lei, J.D. Browning, P.A. Eichen, et al., Immuno-stimulatory activity of a polysaccharide-enriched fraction of Sutherlandia frutescens occurs by the toll-like receptor-4 signaling pathway, J. Ethnopharmacol. 172 (2015) 247–253.
[16] Y. Kanno, A. Ishisaki, E. Kawashita, H. Kuretake, K. Ikeda, O. Matsuo, uPA attenuated LPS-induced inflammatory osteoclastogenesis through the plasmin/PAR-1/Ca(2+)/CaMKK/AMPK axis, Int. J. Biol. Sci. 12 (1) (2016) 63–71.
[17] S. Jeon, S.H. Kim, S.Y. Shin, Y.H. Lee, Clozapine reduces Toll-like receptor 4/NF-κB-mediated inflammatory responses through inhibition of calcium/calmodulin-dependent Akt activation in microglia, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 81 (2018) 477–487.
[18] G. Xu, Y. Li, K. Yoshimoto, et al., 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced inflammatory activation is mediated by intracellular free calcium in microglial cells, Toxicology 308 (2013) 158–167.
[19] A. Belmeguenai, L. Desrues, J. Leprince, H. Vaudry, M.C. Tonon, E. Louiset, Neurotensin stimulates both calcium mobilization from inositol trisphosphate-sensitive intracellular stores and calcium influx through membrane channels in frog pituitary melanotrophs, Endocrinology 144 (12) (2003) 5556–5567.
[20] E.C. Lin, C.L. Combe, S. Gasparini, Differential contribution of Ca(2+)-dependent mechanisms to hyperexcitability in layer V neurons of the medial entorhinal cortex, Front. Cell. Neurosci. 11 (2017) 182.
[21] S. Mukherjee, E.A. Ayaub, J. Murphy, et al., Disruption of calcium signaling in fibroblasts and attenuation of bleomycin-induced fibrosis by nifedipine, Am. J. Respir. Cell Mol. Biol. 53 (4) (2015) 450–458.
[22] D.K. Heo, H.M. Lim, J.H. Nam, M.G. Lee, J.Y. Kim, Regulation of phagocytosis and cytokine secretion by store-operated calcium entry in primary isolated murine microglia, Cell. Signal. 27 (1) (2015) 177–186.
[23] S. Zhang, S. Nie, D. Huang, J. Huang, Y. Feng, M. Xie, Ganoderma atrum polysaccharide evokes antitumor activity via cAMP-PKA mediated apoptotic pathway and down-regulation of Ca(2+)/PKC signal pathway, Food Chem. Toxicol. 68 (2014) 239–246.
[24] Q. Zhang, R. Cong, M. Hu, Y. Zhu, X. Yang, Immunoenhancement of edible fungal polysaccharides (Lentinan, Tremellan, and Pachymaran) on cyclophosphamide-induced immunosuppression in mouse model, Evid. Based Complement. Alternat. Med. 2017 (2017) 9459156.
[25] T. Long, Z. Liu, J. Shang, et al., Polygonatum sibiricum polysaccharides play anticancer effect through TLR4-MAPK/NF-κB signaling pathways, Int. J. Biol. Macromol. 111 (2018) 813–821.
[26] D.D. Wang, W.J. Pan, S. Mehmood, X.D. Cheng, Y. Chen, Polysaccharide isolated from Sarcodon aspratus induces RAW264.7 activity via TLR4-mediated NF-κB and MAPK signaling pathways, Int. J. Biol. Macromol. 120 (Pt A) (2018) 1039–1047.
[27] D. Bi, B. Yu, Q. Han, et al., Immune activation of RAW264.7 macrophages by low molecular weight fucoidan extracted from New Zealand Undaria pinnatifida, J. Agric. Food Chem. 66 (41) (2018) 10721–10728.
[28] Y. Yao, J. Yao, Z. Du, P. Wang, K. Ding, Structural elucidation and immune-enhancing activity of an arabinogalactan from flowers of Carthamus tinctorius L, Carbohydr. Polym. 202 (2018) 134–142.
[29] D.Y. Lee, M.S. Shin, K.S. Shin, Characterization of macrophage-activating polysaccharide isolated from fermented brown rice, J. Med. Food 19 (12) (2016) 1147–1154.
[30] X. Li, W. Xu, TLR4-mediated activation of macrophages by the polysaccharide fraction from Polyporus umbellatus(pers.) fries, J. Ethnopharmacol. 135 (1) (2011) 1–6.
[31] Y. Geng, L. Xing, M. Sun, F. Su, Immunomodulatory effects of sulfated polysaccharides of pine pollen on mouse macrophages, Int. J. Biol. Macromol. 91 (2016) 846–855.
[32] J.Y. Lee, Y.J. Kim, H.J. Kim, Y.S. Kim, W. Park, Immunostimulatory effect of laminarin on RAW 264.7 mouse macrophages, Molecules 17 (5) (2012) 5404–5411.
[33] M.Q. Le, M.S. Kim, Y.S. Song, W.N. Noh, S.C. Chun, D.Y. Yoon, Water-extracted Ampelopsis brevipedunculata downregulates IL-1β,CCL5, and COX-2 expression via inhibition of PKC-mediated JNK/NF-κB signaling pathways in human monocytic cells, J. Pharmacol. Sci. 126 (4) (2014) 359–369.
[34] J.C. Lee, P.R. Young, Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms, J. Leukoc. Biol. 59 (2) (1996) 152–157.
[35] C.C. Franklin, A.S. Kraft, Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells, J. Biol. Chem. 272 (27) (1997) 16917–16923.
[36] C.A. Hazzalin, L.C. Mahadevan, MAPK-regulated transcription: a continuously variable gene switch, Nat. Rev. Mol. Cell Biol. 3 (1) (2002) 30–40.