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Anti-inflammatory Effects of Moutan Cortex Radicis Extract, Paeoniflorin and Oxypaeoniflorin through TLR Signaling Pathway in RAW264.7 Cells

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Abstract Moutan cortex radicis (MCR), the root bark of *Paeonia suffruticosa*, has been widely used as a traditional herb. In this study, we evaluated whether the MCR extract and two active compounds of the bark, paeoniflorin (paeo) and oxypaeoniflorin (oxypaeo), alleviate lipopolysaccharide (LPS)-induced inflammatory responses in RAW264.7 cells and whether they controlled TLR signaling pathway. RAW264.7 cells were treated with the MCR extract or two active compounds in the presence or absence of LPS. The extract and two active compounds inhibited LPS-stimulated nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) gene expression. Additionally, the extract and two active compounds suppressed inflammatory cytokine secretion and gene expression in LPS-stimulated cells. The extract and two active compounds alleviated NF-κB activation by regulating upstream genes in TLR signaling pathway. In addition, the extract and two active compounds decreased phosphorylation of ERK and p38 MAPK. These results indicate that the MCR extract, paeo and oxypaeo have anti-inflammatory effects through regulation of TLR signaling pathway in RAW264.7 cells.

Keywords: *Moutan cortex radicis extract, paeoniflorin, oxypaeoniflorin, inflammation, TLR4, NF-κB, IRF3, MAPKs*

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1. Introduction

Inflammation is a protective response of the body and is caused by harmful stimuli [1]. In general, inflammation has beneficial effects through appropriate regulation. However, control failure caused by some stimuli induces inflammation related diseases such as cancer, atherosclerosis, rheumatoid arthritis (RA) and asthma [2,3]. The appropriate regulation of pattern recognition receptors (PPRs), including trans-membrane proteins such as toll-like receptors (TLRs), c-type lectin receptors (CLRs), and NOD-like receptors (NLRs), is considered as an effective strategy for the reduction of the inflammatory responses [4].

Especially, TLRs are responsible for sensing various ligands such as double-stranded RNA (dsRNA), flagellin and lipoprotein [5]. TLR4 is a member of TLR family and binds to gram-negative bacteria such as lipopolysaccharide (LPS). The activation of TLR4 by LPS stimulates the production of inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor superfamily-α (TNF-α) [6]. This delivers a signal via two different pathways, myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways, within the cell [7,8]. The MyD88-dependent pathway activates nuclear factor kappa B (NF-κB) through expression of the MyD88. The MyD88-independent pathway leads to transcription of interferon regulatory factor 3 (IRF3) in the nucleus through the activation of the TIR-domain containing adapter inducing interferon β (TRIF) and the TRIF-related adaptor molecule (TRAM).

Activation of mitogen-activated protein kinases (MAPKs), including p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun amino-terminal kinase (JNK), leads to inflammatory responses in inflammation-associated diseases [9,10]. MAPKs are also related to NF-κB subunit p65 transactivation [11]. Therefore, regulation of MAPKs plays an important role in the inhibition of inflammatory responses.

In recent studies, various phytochemicals have been demonstrated to have a beneficial effect on inflammation-associated diseases [1,12]. Moutan cortex radicis (MCR) is widely used as a traditional herb in the East, and it has anti-oxidation properties and decreases periodontal diseases [13,14]. Recently, several studies have shown that paeoniflorin (paeo) and oxypaeoniflorin (oxypaeo), major constituents of MCR, have neuroprotective effects and are anti-oxidants [15,16]. However, the MCR extract and two active compounds, paeo and oxypaeo, have not been clearly evaluated for their anti-inflammatory effects

on LPS-stimulated RAW264.6 cells. Therefore, we examined whether the MCR extract inhibits LPS-induced inflammatory responses through regulation of TLR signaling pathway. Additionally, we confirmed that paeo and oxypaeo have important roles in the MCR extract.

2. Materials and Methods

2.1. Materials and Reagents

MCR was obtained from Kangwon National University (St. Gangwondaehak, Chuncheon,, South Korea). Paeoniflorin (paeo) and oxypaeoniflorin (oxypaeo) were purchased from MedChem Express (Princeton, NJ, USA). RAW264.7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's media (DMEM), fetal bovine serum (FBS), 100 units/ml penicillin streptomycin, and 100 μg/ml streptomycin were purchased from Gibco (Grand Island, NY, USA). Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was purchased from RMS Bio-solution (Seoul, Korea). Thiazolyl Blue tetrazolium bromide (MTT) was purchased from Alfa Aesar (Ward Hill, MA, US). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from BioLegend (San Diego, CA, USA). TRIzol® reagent was purchased from Invitrogen (Carlsbad, CA, USA). Primers were purchased from Bioneer (Daejeon, South Korea). Antibodies were purchased from Cell Signaling Technology (Boston, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Preparation Process of the MCR Extract

We used the MCR plants at six years of age for the experiment. The soil on the MCR was removed by washing with water. After air drying, it was cut into small pieces and extracted with hot water for 3 h. Next, the extract was filtrated through Whatman no. 5 filter paper and concentrated by vacuum furnace. The concentrated extract was freeze-dried at −45°C under vacuum for at least 48 h and was stored at 4°C until use.

2.3. RAW264.7 Cell Culture and Viability

RAW264.7 cells, a murine macrophage cell line, were cultured at 37 °C in DMEM supplemented with 10 % FBS and 1 % penicillin streptomycin. To evaluate cell viability, RAW264.7 cells (5×10^3 cells/well) were cultured in 96-well plate. Cells were exposed to the presence or absence of the MCR extract, paeo, and oxypaeo for 24 h.

2.4. Measurement of Nitric Oxide Content

RAW264.7 cells were seeded into 12-well plates at a density of 1×10^5 cells/well. RAW264.7 cells were treated with the MCR extract (0, 50, and 100 μg/mL), paeo (0, 10, and 30 μM), and oxypaeo (0, 10, and 30 μM) for 4 h. Cells were treated with LPS (1 μg/mL) and incubated for 18 h at 37 °C in a CO_2 incubator. After the various treatments, the cell culture medium (100 μL) was mixed with Griess reagent (100 μL) and incubated at room temperature for 20 min. The NO concentration was determined at 540 nm using $NaNO_2$ as a standard.

2.5. Evaluation of Inflammatory Cytokines Secretion in RAW264.7 Cells

RAW264.7 cells were pre-treated with the three compounds for 4 h and then stimulated with or without LPS (1 μg/mL) for 18 h. Cell supernatants were thawed only once, immediately before performing the cytokine assay. We measured the cytokine levels (TNF-α, IL-1β, and IL-6) in supernatants using ELISA kits as per the manufacturer's instructions.

2.6. RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNAs were extracted using TRIzol® reagent and subsequently used to generate cDNA using an RT-PCR system. Target gene amplification was performed using specific oligonucleotide primers in a normal PCR system. The forward and reverse sets of RT-PCR primers were designed as follows: TNF-α, forward (5′-CTACTCCT CAGAGCCCCCAG-3′) and reverse (5′-TGACCACTC TCCCTTTGCAG-3′); IL-1β, forward (5′-CAGGATGAG GACATGAGCACC-3′) and reverse (5′-CTCTGCACAC TCAAACTCCAC-3′); IL-6, forward (5′-CCATCTCTCC GTCTCTCACC-3′) and reverse (5′-AGACCGCTGCCT GTCTAAAA-3′); IL-10, forward (5′-CAGTACAGCCG GGAAGACAA-3′) and reverse (5′-TCCAGCTGGTCCT TTGTTTG-3′); MyD88, forward (5′-ACTGGCCTGAGC AACTAGGA-3′) and reverse (5′-CGTGCCACTACCT GTAGCAA-3′); IRAK4, forward (5′-AGCTGCGTCACC TACCTGTT-3′) and reverse (5′-GTTTGGTGATGTTG CTGTGG-3′); were used. The PCR product was separated on 1.5 % agarose gel and visualized by UV after ethidium bromide staining.

2.7. Western Blot Analysis

After RAW264.7 cells were washed using 1 × PBS, cells were lysed by lysis buffer (including phosphatase inhibitor cocktail 2 and phosphatase inhibitor cocktail 3). Protein content was determined using the Bradford assay. Protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immune-Blot PVDF membrane, Bio-Rad). Membranes were immunoblotted with primary antibodies specific for iNOS, p-TBK1, TBK1, p-IRF3, IRF3, p-IKK, IKK, p-IκBα, IκBα, p-NF-κB p65, NF-κB p65, p-ERK, ERK, p-JNK, JNK, p-p38, p38 and α-tubulin at 4°C overnight. Membranes were then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000) for 2 h. Bands were visualized using an enhanced chemiluminescence system (ECL, Thermo Fisher Scientific) and LAS image software (Fuji, New York, NY, USA).

2.8. Statistical Analysis

Differences among multiple groups were determined by a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, using the SPSS software

system (SPSS for Windows, version 20; SPSS, Inc., Chicago, IL). Values marked with different letters are significantly different, $p<0.05$.

3. Results and Discussion

3.1. Evaluation of Cell Viability Assay Results of the MCR Extract, Paeo, or Oxypaeo on RAW264.7 Cells

Prior to investigating the MCR extract, paeo, or oxypaeo on LPS-induced inflammation in RAW264.7 cells, we performed a cell viability assay to select the proper concentrations of the MCR extract and two compounds for further investigation. Cells were treated with increasing concentrations of the MCR extract (0, 6.3, 12.5, 25, 50, and 100 μg/mL) and two compounds (0, 10, 20, and 30 μM) for 24 h. We observed that the MCR extract and two compounds were not toxic to cells (Figure 1). Therefore, a range of concentrations of the MCR extract (0, 50, and 100 μg/mL) and two compounds (0, 10, and 30 μM) was selected for further experiments.

3.2. Effect of the MCR Extract, Paeo, or Oxypaeo on the Production of Nitric Oxide (NO) and on the Protein Expression of Inducible Nitric Oxide Synthase (iNOS) in LPS-induced RAW264.7 Cells

To determine the effect of the MCR extract, paeo, or oxypaeo on LPS-induced inflammation, cells were stimulated with LPS in the presence or absence of the MCR extract, paeo, or oxypaeo for 18 h. The production of NO is one of the markers of LPS-induced inflammation [17]. As shown in Figure 2A, LPS stimulated the production of NO in RAW264.7 cells, and the MCR extract and two compounds inhibited the production of NO. Additionally, the MCR extract and two compounds suppressed the mRNA expression and protein levels of iNOS, which is a NO production-associated protein, compared to the LPS-treated cells (Figure 2B, Figure 2C). Thus, the MCR extract, paeo, and oxypaeo decreased LPS-induced NO production by regulating iNOS gene expression.

3.3. The Production of Pro-inflammatory Cytokines such as TNF-α, IL-1β, and IL-6 was Suppressed by the MCR Extract, Paeo, or Oxypaeo in LPS-induced RAW264.7 Cells

It is well known that LPS activates TLR signaling pathway and subsequently produces pro-inflammatory cytokines in macrophage cells [18]. To determine the effect of the MCR extract, paeo, or oxypaeo on pro-inflammatory cytokines, cells were stimulated with LPS in the presence or absence of the MCR extract, paeo, or oxypaeo for 18 h. As shown in Figure 3A-C, it was observed that LPS increased the production of TNF-α, IL-1β, and IL-6 when compared to cells treated with the absence of LPS, while the MCR extract, paeo, or oxypaeo decreased LPS-induced production of TNF-α, IL-1β, and IL-6 compared to the corresponding control. In addition, the extract and two active compounds inhibited pro-inflammatory cytokines gene expression (Figure 3D). These results show that the MCR extract, paeo, and oxypaeo decreased secretion of LPS-induced inflammatory cytokines through regulation of pro-cytokine gene expression in cell.

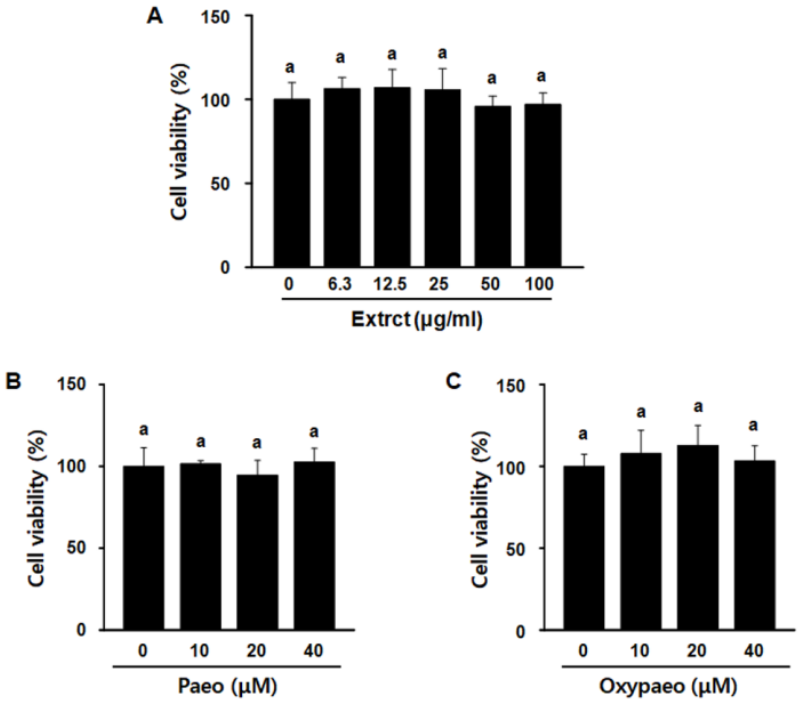

Figure 1. Effect of the MCR extract, paeoniflorin, and oxypaeoniflorin on the cytotoxicity of RAW264.7 cells. Viability of cells treated for 24 h with the MCR extract, paeoniflorin, and oxypaeoniflorin. Absorbance at 570 nm was recorded in an ELISA plate reader. (A) the MCR extract; (B) paeoniflorin; (C) oxypaeoniflorin. Values with different letters are significantly different, $p < 0.05$. The experiment was performed in hexaplicate

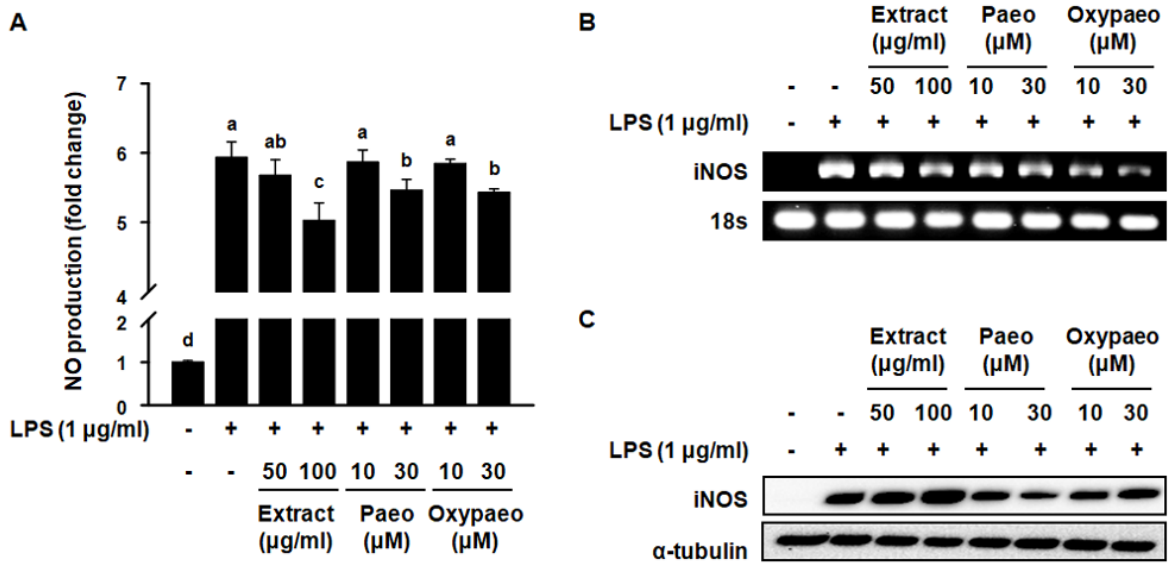

Figure 2. Effect of the MCR extract, paeoniflorin, and oxypaeoniflorin on nitric oxide (NO) production and protein expression of iNOS in LPS-induced RAW264.7 cells. Cells were pre-treated with the MCR extract (50 and 100 μg/ml), paeoniflorin (10 and 30 μM), and oxypaeoniflorin (10 and 30 μM) for 4 h and then co-treated with LPS (1 μg/ml) for 18 h. (A) the production of NO in LPS-induced RAW264.7 cells with the presence or absence of the MCR extract, paeoniflorin and oxypaeoniflorin using the Griess reagent at 540 nm. (B) The expression of iNOS mRNA using RT-PCR. (C) The expression of iNOS protein using western blot. Values with different letters are significantly different, $p < 0.05$. The experiment was performed in triplicate

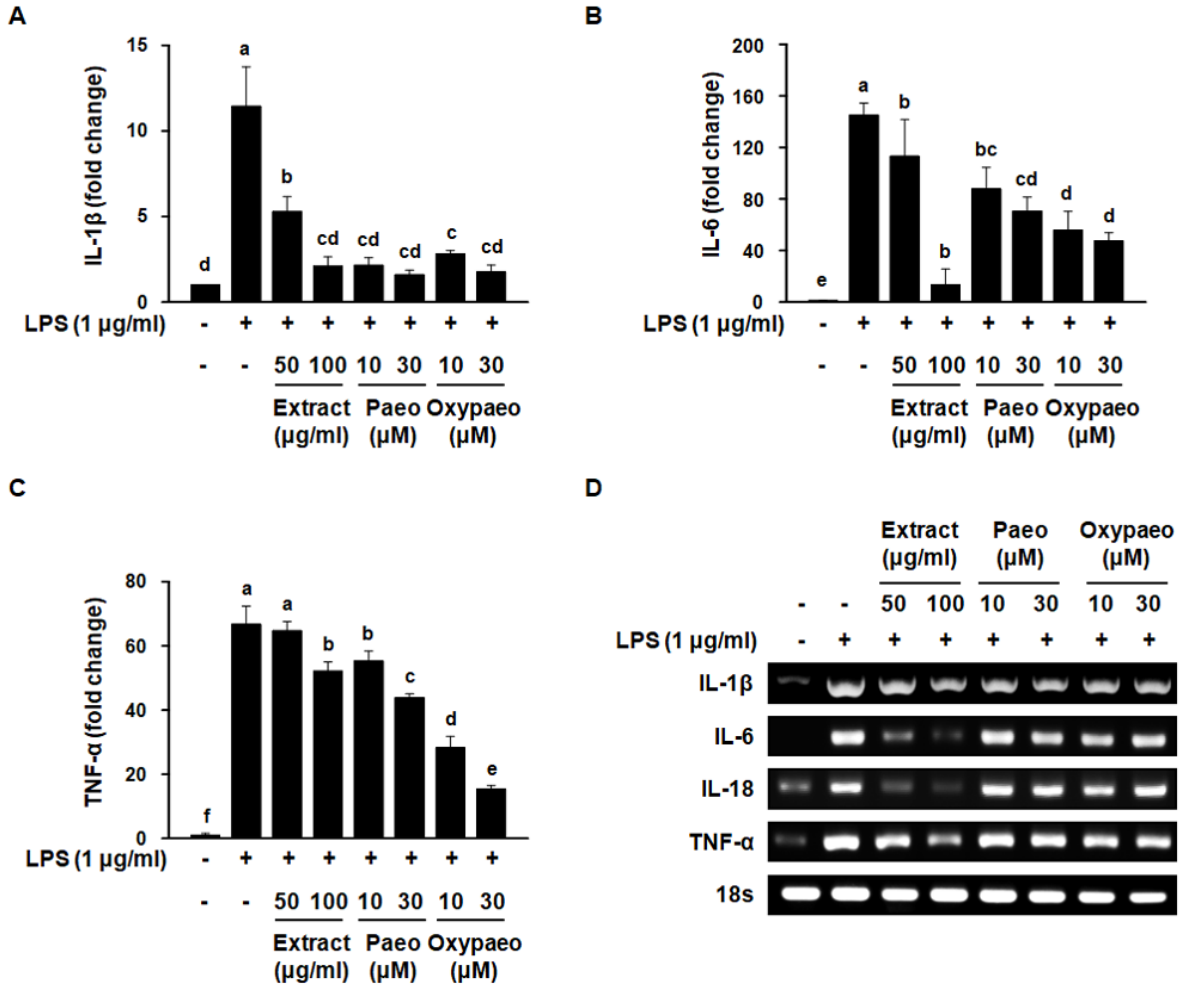

Figure 3. The MCR extract, paeoniflorin, and oxypaeoniflorin suppress the production of inflammatory cytokines and their mRNA expression in LPS-induced RAW264.7 cells. Cells were pre-treated with the MCR extract, paeoniflorin, or oxypaeoniflorin for 4 h and then stimulated for 18 h with LPS (1 μg/ml). The production of inflammatory cytokines in supernatants was measured using ELISA kit at 450 nm and 560 nm. (A) The production of IL-1β; (B) The production of IL-6; (C) The production of TNF-α. (D) The mRNA expression of pro-inflammatory cytokines was measured by RT-PCR. Values with different letters are significantly different, $p < 0.05$. The experiment was performed in tetraplicate and triplicate

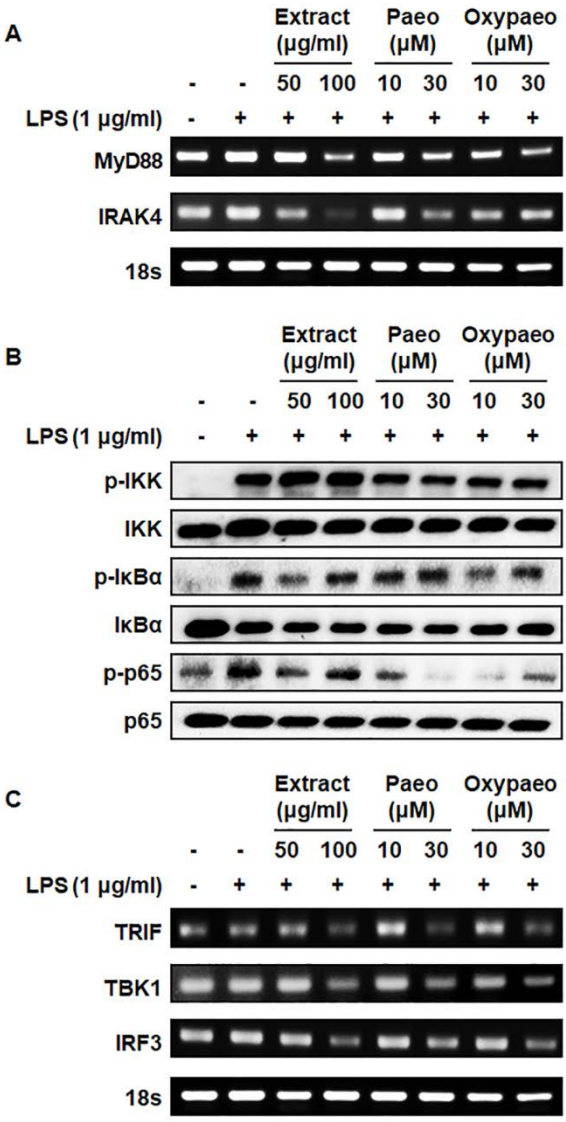

Figure 4. The MCR extract, paeoniflorin, and oxypaeoniflorin attenuate the downstream targets of TLR4 signaling pathway in LPS-induced RAW264.7 cells. Cells were pre-treated with the MCR extract, paeoniflorin, or oxypaeoniflorin for 4 h and then stimulated with LPS (1 μg/ml). The mRNA expression of pro-inflammatory cytokines was measured by RT-PCR. The protein level of MAPKs was measured using western blot. The experiment was performed in triplicate

3.4. Effect of the MCR Extract, Paeo, or Oxypaeo on Downstream Target of TLR Signaling Pathway

We showed that the MCR extract, paeo, and oxypaeo suppress inflammatory responses of LPS–stimulated cells. To understand the molecular mechanism underlying the anti-inflammation of the MCR extract, paeo, or oxypaeo, we investigated TLR signaling pathway. TLR signaling pathway is an inflammatory response related pathway [19]. In many studies, regulation of TLR signaling pathway is one of the strategic methods to alleviate inflammatory responses [20,21]. We indicated a capacity of the extract and two compounds to control TLR signaling pathway. The MCR extract, paeo and oxypaeo inhibited the expression of MyD88 and IRAK4 genes, the constituents of the MyD88-dependented pathway, gene expression (Figure 4A). Additionally, the MCR extract, paeo and oxypaeo decreased LPS-stimulated TRIF, TBK1 and IRF3 gene expression, constituents of the MyD88-independent pathway (Figure 4C). Next, we studied whether the extract and two active compounds decreased LPS-induced NF-κB activation. NF-κB activation induced inflammatory responses, such as inflammatory cytokine secretion and the production of NO [22]. As shown in Figure 4B, the MCR extract inhibited LPS-stimulated NF-κB activation at a low dose through regulation of the phosphorylation of IκBα. Paeo decreased NF-κB activation, whereas IKK and IκBα were not affected. Oxypaeo suppressed NF-κB activation by regulating the phosphorylation of IκBα in LPS-stimulated cells. Therefore, the MCR extract, paeo and oxypaeo had anti-inflammatory effects through targeting TLR signaling pathway.

3.5. The MCR Extract, Paeo, or Oxypaeo Attenuates a part of the MAPK signaling Pathway in LPS-induced RAW264.7 Cells

MAPKs are related to the extracellular signals that play important roles in inflammatory responses [23]. We investigated whether the MCR extract, paeo and oxypaeo attenuate the phosphorylation of MAPKs. As shown in Figure 5, the MCR extract and paeo regulated the phosphorylation of ERK and p38 MAPK in LPS-stimulated cells. Oxypaeo only suppressed the phosphorylation of p38 MAPK, unlike extract and paeo. The phosphorylation of JNK was not affected by the extract, paeo, or oxypaeo. These results showed that the MCR extract, paeo, and oxypaeo partially affected LPS-induced phosphorylation of MAPKs in RAW264.7 cells.

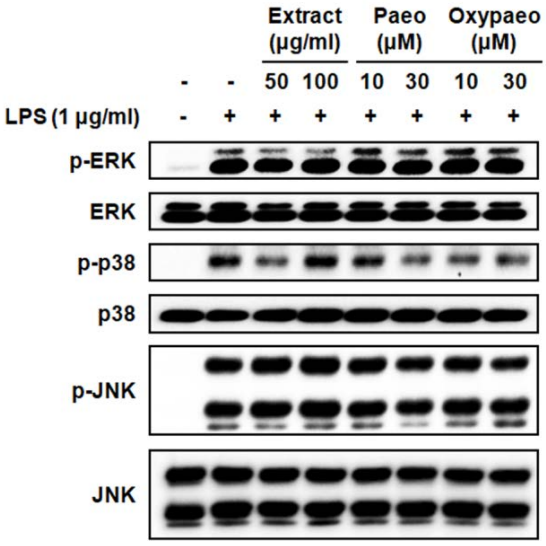

Figure 5. The MCR extract, paeoniflorin, and oxypaeoniflorin attenuate LPS induced the phosphorylation of MAPKs in RAW264.7 cells. Cells were pre-treated with the MCR extract, paeoniflorin, and oxypaeoniflorin for 4 h and then stimulated for 30 min with LPS (1 μg/ml). The protein levels of MAPKs were measured using western blot. The experiment was performed in triplicate

4. Conclusions

Taken together, we have shown here that the MCR extract, paeo, and oxypaeo alleviate LPS-induced inflammatory

responses through the regulation of TLR signaling pathway in RAW264.7 cells. In addition, the MCR extract, paeo, and oxypaeo partially suppressed the phosphorylation of MAPKs, ERK and p38 MAPK. These findings suggested that the MCR extract may function as a potential natural bioactive compound with anti-inflammatory effects. Also, paeo and oxypaeo may also function as active components from the MCR extract.

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Conflicts of Interest

The authors declare no conflict of interest.

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