적작약 추출물의 대식세포에 대한 염증억제 효과

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Suppression Effect of the Inflammatory Response in Macrophages by Paeoniae Radix Rubra Extracts

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ABSTRACT : Paeoniae Radix Rubra is a preparation consisting of desiccated roots of *Paeonia lactiflora* PALL (belonging to *Ranunculaceae*). Paeoniae Radix Rubra is used as a medicinal herb in Asian countries to treat many diseases. Ethanol- or water-based extracts of Paeoniae Radix Rubra were prepared and tested on RAW 264.7 cells, a murine macrophage cell line. The expression of some pro-inflammatory proteins, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphorylated ERK1/2 was detected by Western blot analyses, while PGE2 expression was quantified by ELISA. Both the water and ethanol extracts of Paeoniae Radix Rubra suppressed LPS-induced nitric oxide (NO) production and exhibited cell toxicity in accordance with increased NO production. Also, both extracts reduced the expression of COX-2 and iNOS, and inhibited phosphorylation of ERK1/2 in LPS-stimulated RAW 264.7 cells. Extracts prepared from Paeoniae Radix Rubra contain anti-inflammatory agents that inhibit the iNOS and MAPK pathways.

***Key Words* : Paeoniae Radix Rubra, iNOS, COX-2, ERK1/2, PGE2**

INTRODUCTION

Inflammation is implicated in the pathogenesis of many diseases and is involved in the production of inflammation mediators and cytokines such as reactive oxygen species (ROS), reactive nitrogen species, nitric oxide, and prostaglandin E_2 (PGE_2) (Hibbs *et al*., 1987; Palmer *et al*., 1988; Kock *et al*., 1990; Lowenstein *et al*., 1996; Lawrence *et al*., 2002). Macrophages play a central role in the inflammatory response and serve as an essential interface between innate and adaptive immunity (Adams and Hamilton, 1984). Macrophages exert their anti-microbial effects directly through phagocytosis (Aderem, 2003). During the inflammatory response, when macrophages are exposed to lipopolysaccharide (LPS) or interferon-γ (IFN-γ), they are activated and release pro-inflammatory mediators and cytokines including NO, ROS and PGE_2 (Nathan, 1987; Wu *et*

al., 2008). Overproduction of inflammation mediators causes many diseases such as asthma, Alzheimerís disease, and atopic dermatitis (Chan *et al*., 1993; Mahut *et al*., 2004; Culbert *et al*., 2006; Puckett *et al*., 2010). Previous studies have demonstrated that inhibition of pro-inflammatory mediators by macrophages could attenuate the severity of these disorders.

Nitric oxide (NO) is not only a diffusible intercellular molecule but also an intracellular signalling molecule (Schmidt and Walter, 1994). NO has diverse effects on the physiological function of smooth muscle cells, neurons, platelets and immune cells. Nitric oxide synthases (NOS) generate NO by catalyzing the oxidation of guanidine-nitrogen of L-arginine (Bredt and Snyder, 1989; Palmer and Moncada, 1989). NOS have three different isoforms that are expressed in a tissue-specific manner: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Sessa, 1994). nNOS and eNOS are stimulated to synthesize NO

by the calmodulin signaling pathway (Bredt and Snyder, 1994). iNOS is normally not detected; however, iNOS can be up-regulated in a variety of tissues and cells after stimulation by lipopolysaccharide (LPS) or cytokines (Adams *et al.*, 1997; Torres *et al.*, 2004). A high level of NO production from iNOS as part of the inflammatory response is detrimental for cell viability and function (Skidgel *et al.*, 2002). NO is a free radical and its cytotoxic effects are due to peroxynitrite (OONO⁻) formation and nitration of tyrosine residues in proteins, or generation of superoxide (Lipton *et al.*, 1993).

Cyclooxygenase (COX), a prostaglandin (PG) synthase, is the rate-limiting step in the biosynthesis of biologically active and physiologically important prostaglandins (Wu, 1996). There are two isoforms of COX, COX-1 and COX-2; a third possible isoform, COX-3, is a splice variant of COX-1. COX-1 is constitutively expressed in most tissues but, in contrast to COX-2, is expressed in low or undetectable levels in the resting state of cells (Tetsuka *et al.*, 1996; Williams *et al.*, 1996).

COX-2 expression is induced by inflammatory cytokines and tumor promoters and is regulated by several pathways (Schneider and Stahl, 1998) and both the Akt and MAPK pathways seem to play an important role (McGinty *et al.*, 2000). ERK1/2 has important role in MAPK pathways and activation of ERK1/2 was reported regulation of COX-2 expression in human mesangial cells (HMC) (Rodriguez-Barbero *et al.*, 2006). Therefore, it has possibility that activation of ERK1/2 is necessary for COX-2 expression in LPS-stimulated RAW264.7.

Recently, anti-inflammatory effect of plants resource was attracted (Yoon *et al.*, 2010; Lee *et al.*, 2011). Paeoniae Radix Rubra, a combination of desiccated roots of *Paeonia lactiflora* PALL (belonging to *Ranunculaceae*), has been used as a medicinal herb in traditional Asian medicine for treating blood stasis, pain relief, and for treating cardiovascular, inflammatory, and female reproductive diseases (Wu *et al.*, 2010). Recently, studies have reported that NO production is suppressed in the injured lung by Paeoniae Radix Rubra (Zhan *et al.*, 2006; Chen *et al.*, 2008). Also it protects the liver from BCG endotoxin-induced injury by down-regulating production of pro-inflammatory cytokines (Sun *et al.*, 2008). In this study, we prepared two types of extracts from Paeoniae Radix Rubra, using either water or ethanol. Then we evaluated the effect of each extract for anti-inflammatory activity and cytotoxicity. We found that Paeoniae Radix Rubra has a role as an anti-inflammatory in several signalling pathways including iNOS, COX-2, and ERK1/2 in LPS-stimulated RAW 264.7cells.

MATERIALS AND METHODS

1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The prostaglandin E_2 (PGE_2) ELISA assay was from R&D Systems (Minneapolis, MN, USA). Anti-iNOS, anti-COX-2, anti-ERK1/2, and anti-phosphorylated ERK1/2 mouse or rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Preparation of Plant Extracts

The sliced dried roots of Paeoniae Radix Rubra was purchased from HMAX (Jecheon, Korea), and was authenticated by Professor Ee Hwa Kim, the School of Oriental Medicine, Semyung University. 1 kg dried roots were extracted with 5 L distilled water at 95℃ for 3 h under heating mantle-reflux three times. The extract was filtered through no. 2 filter paper (Advantec, Japan) and evaporated under reduced pressure at in a vacuum rotatory evaporator (Buchi, German). Afterwards, the concentrated water extract was freeze-dried by FDS lyophilizer system (Ilsin, Korea). The water extract (yield, 28.5 g/kg) was kept refrigerated at 4℃. 1 kg dried roots were extracted with 5 L absolute ethanol at room temperature for 48 h under overhead stirrer three times. The extract solution was filtered and then evaporated under reduced pressure. The ethanol extract (yield, 32.5 g/kg) was kept refrigerated at 4℃.

3. Cell Culture

The RAW 264.7 murine macrophage cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (100 U/mℓ penicillin, 100 ㎎/mℓ streptomycin; Invitrogen) in an incubator at 37℃ in 5% CO_2.

4. Nitric Oxide Determination

NO accumulation was used as an indicator of NO production in the cell culture medium and was measured using the Griess reaction. The cells were cultured at a density of 1.0×10^5 cells per well in a 96-well plate format. At 16 hours after stimulating the cells with 1 ㎍/mℓ of LPS, the cell culture supernatant (100 ㎕) was mixed with an equal volume of Griess reagent [1% sulphanilamide and 0.1% N-(1-naphtyl)-ethylenediamine dihy-

drochloride in 5% phosphoric acid] for 10 minutes, and the absorbance was measured at 540 ㎚. Sodium nitrite ($NaNO_2$) was used to generate a standard curve (1-100 μM).

5. MTT Assay

RAW 264.7 cells (1.0×10^4) were seeded in each well of a 96-well plate and incubated for 16 hours at 37℃ in 5% CO_2. The cells were then exposed to various concentrations of Paeoniae Radix Rubra extract for 24 hours followed by incubation in formazan substrate for an MTT assay. After 2 hours of incubation at 37℃, the supernatant was discarded and 100 ㎕ of DMSO was added. The optical density was measured at 570 ㎚ using the SpectraMax190 spectrophotometer (Molecular Devices; Sunnyvale, CA, USA). Cell survival rates were expressed as a percentage of the value of the medium group.

6. Immunoblot

RAW 264.7 cells (1.0×10^6 cells/well) were plated overnight in a 6-well culture plate. Cells were further incubated in DMEM without L-glutamine for 24 hours. Cells were washed and lysed in homogenizing radioimmunoprecipitation (RIPA) buffer containing protease inhibitors. The total cell lysates were prepared and 25 ㎍ of soluble proteins were separated by sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking for non-specific antibody binding (5% bonive serum albumin (BSA) in tris buffered saline tween20 (TBS-T)), the membranes were incubated with antibodies against COX-2 (1 : 1000 dilution), iNOS (1 : 1000), Erk1/2 (1 : 1000) or phosphorylated Erk1/2(1 : 1000). After several washes in TBS-T, the membranes were probed for secondary antibodies conjugated to horseradish peroxidase against mouse or rabbit IgG (1:20000 dilution) for 1 hour at room temperature. Following three washes in TBS-T, immunoreactive bands were visualized using the Oddyssey Imager (LI-COR; Lincoln, Nebraska, USA).

7. Measurement of PGE_2

Quantitation of PGE_2 secretion was performed by ELISA. RAW 264.7 cells (1.0×10^4 cells/well) were cultured in 96 well plates and incubated in the presence or absence of LPS (1 ㎍/㎖) for 16 hours. Then, the cell culture supernatant was collected to determine PGE_2 concentration. ELISA was performed according to the manufacturerís instruction and quantitated with a SpectraMax190 spectrophotometer (Molecular Devices, USA). Values from the proliferation assays were used for standardization.
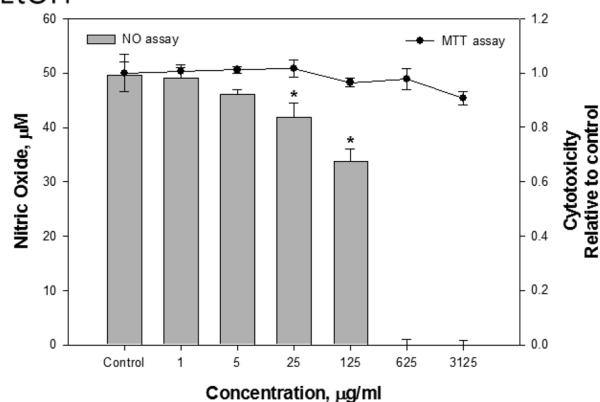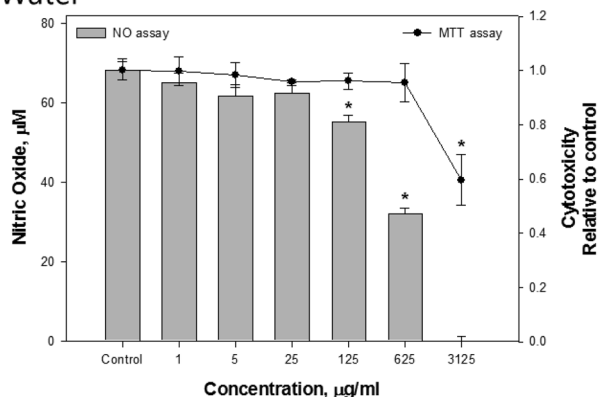

Fig. 1. Inhibitory effect of EtOH and water extracts from Paeoniae Radix on nitric oxide production in LPS-stimulated RAW 264.7 cells. The cells (1.0×10^5 cells/well) were stimulated with 1 ㎍/㎖ of LPS or with LPS plus various concentrations (1, 5, 25, 125, 625, 3125 ㎍/㎖) of ethanol-based or water-based Paeoniae Radix extract for 24 h. Nitric oxide production was determined using the Griess reagent method. Cell viability was determined 24 h after the cells (1.0×10^4 cells/well) were stimulated with LPS (1 ㎍/㎖) and extract. Cell viability was determined using the MTT method. Values (n = 3) are presented as the mean±S.D. of experiments performed in triplicate (*$p < 0.05$).

8. Statistical Analysis

The results obtained were expressed as mean ± S.D. The Studentís t-test was used to make a statistical comparison between the groups and results with $p < 0.05$ were considered to be statistically significant.

RESULTS

1. Effect of Paeoniae Radix Rubra extracts on cellular cytotoxicity and NO production

Our first goal was to assess whether extracts prepared from Paeoniae Radix Rubra are cytotoxic. RAW 264.7 cells were

exposed to different concentrations of the water-based or ethanol-based extracts at 1, 5, 25, 125, 625 or 3125 μg/mℓ. A subsequent MTT assay revealed slight cellular cytotoxicity at a concentration of 3125 μg/mℓ for the ethanol-based extract and significant cytotoxicity at 3125 μg/mℓ for the water-based extract (Fig. 1). To verify anti-inflammatory activity, we measured NO release and found that NO production decreased significantly starting at 25 μg/mℓ for the ethanol-based extract and 125 μg/mℓ for the water-based extract (Fig. 1). Also, the ethanol-based extract appears to be the more potent anti-inflammatory agent as there was much greater inhibition of NO production with the ethanol-based Paeoniae Radix Rubra treatment.

2. Effect of extracts of Paeoniae Radix Rubra on expression of PGE$_2$

Increased prostaglandin E$_2$ (PGE$_2$) expression is correlated with increased inflammation. Therefore, we investigated whether the Paeoniae Radix Rubra extracts had any effect on PGE$_2$ expression. RAW 264.7 cells were treated with LPS or in combination with 50, 100, 150 or 200 μg/mℓ of the extracts; then the expression of PGE$_2$ was detected by ELISA. Under these conditions, RAW 264.7 cells normally released PGE$_2$. We saw a slight, but significant inhibition of PGE$_2$ production when the cells were treated with 200 μg/mℓ of the water-based extract (Fig. 2). The ethanol-based extract was a more potent inhibitor of PGE$_2$ production as both the 150 and 200 μg/mℓ treatments were able to significantly inhibit PGE$_2$ in LPS-stimulated RAW 264.7cells (Fig. 2). Therefore, although both extracts are able to inhibit PGE$_2$ production, the ethanol-based Paeoniae Radix Rubra extract appears to have the higher potency.

3. Suppression of MAPK pathways by both the aqueous and ethanol extracts of Paeoniae Radix Rubra

LPS-induced RAW 264.7 cells release NO by the action of the iNOS system. Therefore, we determined the expression levels of some common inflammatory molecules when macrophages were treated with the Paeoniae Radix Rubra extracts. COX-2 and iNOS expression were induced by LPS stimulation and were suppressed when treated with the extracts at a concentration of 150 and 200 μg/mℓ (Fig. 3). Similarly, ERK1/2 becomes phosphorylated upon LPS stimulation and the phosphorylation can be inhibited by treatment with the extracts, especially at the higher concentrations (Fig. 4). Taken together, our data suggest that extracts prepared from Paeoniae Radix Rubra have anti-inflammatory effects by suppressing the expression and or
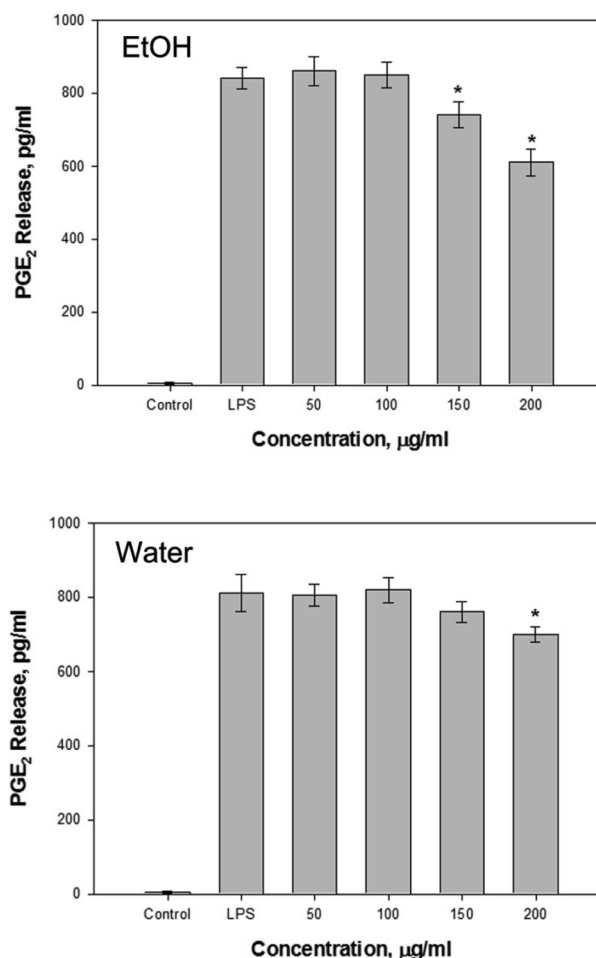

Fig. 2. Inhibitory effect of EtOH and water extracts from Paeoniae Radix on PGE$_2$ production in LPS-stimulated RAW 264.7 cells. The cells (1.0×10^4 cells/well) were stimulated with 1 μg/mℓ of LPS or with LPS plus various concentrations (50, 100, 150, 200 μg/mℓ) of ethanol-based or water-based Paeoniae Radix extract for 16 h. PGE$_2$ production and release into the culture medium was assayed by ELISA. The data (n = 3) represent the mean±S.D. of experiments performed in triplicate (*$p < 0.05$).

function of certain pro-inflammatory molecules. Also, it appears that both the ethanol-based and water-based extracts are nearly equal in their ability to inhibit these pro-inflammatory molecules.

DISCUSSION

Paeoniae Radix Rubra is a preparation of desiccated roots of *Paeonia lactiflora* PALL(belonging to *Ranunculaceae*). Our data demonstrate that Paeoniae Radix Rubra exhibits pharmacological activity via significant inhibitory effects on the LPS-induced inflammatory mediators and cytokines such as NO and PGE$_2$ in RAW 264.7 mouse macrophages. These effects were mediated
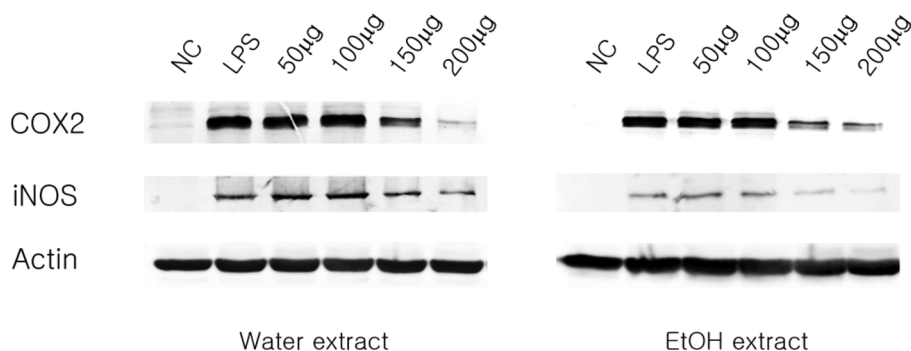
Fig. 3. Inhibitory effects of EtOH and water extracts from Paeoniae Radix on iNOS and Cox2 expression in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/mℓ) were stimulated with LPS (1 μg/mℓ) in the presence of extract (50, 100, 150, 200 μg/mℓ). Whole cell lysates (50 μg) were prepared and subjected to 10% SDS-PAGE. Expression of iNOS, COX-2 and actin were determined by Western blot analysis. Actin was used as a loading control.
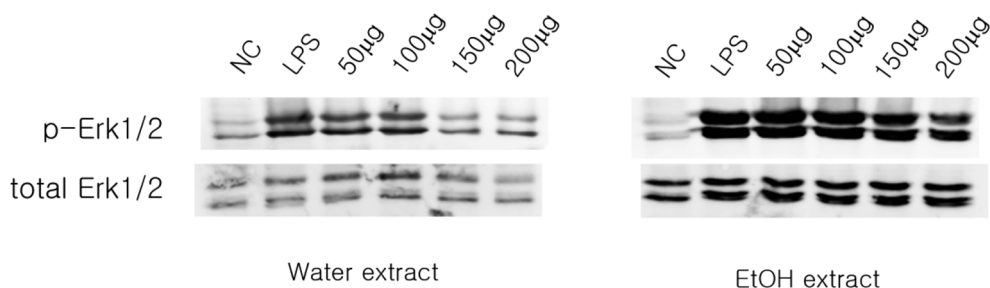

Fig. 4. Inhibitory effect of EtOH and water extracts from Paeoniae Radix on ERK1/2 Phosphorylation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/mℓ) were stimulated with LPS (1 μg/mℓ) in the presence of extract (50, 100, 150, 200 μg/mℓ). Whole cell lysates (50 μg) were prepared and subjected to 10% SDS-PAGE. The expression of phosphorylated ERK1/2 was determined by Western blot analysis and the total cellular ERK1/2 was used as the loading control.

through inhibition of iNOS activation and MAPK pathways.

Macrophages, located in most tissues and organs, have critical roles for immunity such as phagocytosis and secretion of pro-inflammatory cytokines (Adams and Hamilton, 1984; Lowenstein, et al., 1996). Macrophages are activated by bacteria and secrete inflammatory mediators including vascular amines, arachidonic acid metabolites, pro-inflammatory cytokines, PGE$_2$ and reactive oxygen species (ROS). RAW 264.7 cells, a mouse macrophage cell line, generate NO when they are stimulated by cytokines or LPS. Therefore, this study examined the anti-inflammatory activities of Paeoniae Radix Rubra extracts using LPS-challenged RAW 264.7 macrophages as a model system.

Recent studies have reported that plant extracts inhibit the generation of inflammation mediators (NO, PGE$_2$, iNOS and IL-6) from macrophages such as *Astragalus membranaceus*, *Ostericum koreanum* and others (Clement-Kruzel et al., 2008; Jung et al., 2010). Anti-inflammatory effect of leaves and stem from *Paeonia lactiflora* was reported, but the anti-inflammatory effect of root is yet to be reported (Kim et al., 2007). Other studies suggest that LPS significantly activates macrophages and induces COX-2 expression, an enzyme that of converts arachidonic acid into PGE$_2$. Studies have shown that increased COX-2 production is associated with cellular toxicity because inhibition of COX-2 expression and/or activity reduces brain injury after ischemia and slows the progression of Alzheimerís disease and Parkinsonís disease (Grunblatt et al., 2000; Selkoe, 2001; Calderon-Garciduenas et al., 2004). Nitric oxide is highly reactive as a free radical and has an important role physiologically for immune reactions in low concentrations (~nmol range) (Jeremy et al., 1999), but high concentrations (~µmol range) of NO results in many pathological processes including inflammation (Ridnour et al., 2006). It has been reported that iNOS is not normally expressed, but LPS up-regulates iNOS expression in RAW 264.7 cells. Exclusive release of NO from iNOS was correlated with several disorders (Ridnour et al., 2006). Generally, expression of iNOS is induced by immune reactions and plays a role in exacerbating inflammation. In our study, Paeoniae Radix Rubra significantly suppressed

LPS-induced NO production in RAW 264.7 cells in a concentration-dependent manner. This suppression did not show any cytotoxicity at low concentration (until 625 $\mu g/m\ell$).

In addition, PGE_2, an important mediator of the inflammatory response, is synthesized by COX-2 and excess PGE_2 production causes inflammatory diseases such as Alzheimer's disease, Parkinson's disease and colon cancer. Suppression of PGE_2, on the other hand, relieves inflammation and pain. Our results demonstrate that LPS-stimulated PGE_2 production could be suppressed by both Paeoniae Radix Rubra extracts in RAW264.7 cells; however, the decrease was slight. Importantly, this inhibition was concentration-dependent and showed cell-toxicity only in high concentration.

A variety of intracellular signalling pathways are needed to induce and maintain the inflammatory process. MAPK signalling pathway is also involved in the inflammatory process and has been extensively investigated. Recently studies suggested that the MAPK pathways also play a role in regulating PGE_2 expression (Guan *et al*., 1998; Rodriguez-Barbero *et al*., 2006). Therefore, we have demonstrated that activation of MAPK pathways induce COX-2 expression and PGE2 synthesis in LPS-stimulated RAW264.7 cells. Here, we demonstrated that extract prepared from Paeoniae Radix Rubra from 150 $\mu g/m\ell$ inhibit the phosphorylation of ERK1/2, and therefore the MAPK pathway, in a concentration-dependent manner. COX-2 and PGE_2 expression also decreased from 150 $\mu g/m\ell$. These data indicates that the extracts prepared from Paeoniae Radix Rubra have anti-inflammation properties and exert their effects by inhibiting ERK1/2 phosphorylation in LPS-stimulated RAW264.7 cells. In the case of the water-based extract, however, excessive concentration can be cytotoxicity. Therefore, the ethanol-based extract prepared from Paeoniae Radix Rubra, which has a slightly different toxicity but similar efficiency on inflammatory activity, might be the preferred method of preparing extracts of Paeoniae Radix Rubra. Future studies might prove Paeoniae Radix Rubra to be an effective therapy for many other inflammatory diseases.

Acknowledgements

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