Pharmacological Reports
2011, 63, 781–789
ISSN 1734-1140

Puerarin inhibits iNOS, COX-2 and CRP expression *via* suppression of NF-κB activation in LPS-induced RAW264.7 macrophage cells

Wenzhi Hu[1]*, Xiangjun Yang[1]*, Cao Zhe[2], Qin Zhang[3], Lie Sun[2], Kejiang Cao[4]

[1]Department of Cardiology, First Affiliated Hospital of Soochow University, Soochow, China

[2]Department of Cardiology, Second Affiliated Hospital of Nanjing Medical University, Nanjing, China

[3]Osteopathia Laboratory, Department of Orthopaedics, First Affiliated Hospital of Soochow University, Soochow, China

[4]Department of Cardiology, First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Correspondence: Kejiang Cao, e-mail: Caokejiang2010@yahoo.cn

Abstract:
Puerarin (7,4'-dihydroxy-8-C-glucosylisoflavone) is the most abundant isoflavone-C-glucoside extracted from *Radix puerariae*, and it has been used for various medicinal purposes in traditional oriental medicine for thousands of years. In the present study, the ability of the puerarin to modulate inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and C reactive protein (CRP) expression and induce changes in the nuclear factor κB (NF-κB) pathway in RAW264.7 macrophage cells was examined. The protein and mRNA levels of lipopolysaccharide (LPS)-induced iNOS, COX-2 and CRP were determined in RAW246.7 macrophage cells. Inhibitor κB (I-κB) phosphorylation and p65NF-κB expression in RAW246.7 macrophage cells were also detected under our experimental conditions. The results indicated that puerarin inhibited the expression of LPS-induced iNOS, COX-2 and CRP proteins and also suppressed their mRNAs from RT-PCR experiments in RAW264.7 cells. Subsequently, we determined that the inhibition of iNOS, COX-2 and CRP expression was due to a dose-dependent inhibition of phosphorylation and degradation of I-κB, which resulted in the reduction of p65NF-κB nuclear translocation. These data suggested that the effect of puerarin-mediated inhibition of LPS-induced iNOS, COX-2 and CRP expression is attributed to suppressed NF-κB activation at the transcriptional level.

Key words:
puerarin, inducible nitric oxide synthase, cyclooxygenase-2, C reactive protein, nuclear factor-κB, lipopolysaccharide

Abbreviations: COX-2 – cyclooxygenase-2, CRP – C reactive protein, iNOS – inducible nitric oxide synthase, LPS – lipopolysaccharide, NF-κB – nuclear factor-κB

Introduction

During inflammatory disease, the macrophage produces excessive amounts of mediators, such as nitric oxide (NO), prostaglandins (PGs), C reactive protein (CRP) and pro-inflammatory cytokines [14, 21]. Nitric oxide synthase (NOS) and the cyclooxygenase (COX) system play a major role in similar pathophysiological conditions, such as inflammation and cancer [18]. Molecular cloning and sequencing analysis have revealed the existence of at least three main types of NOS isoforms: neuronal NOS, endothelial NOS and inducible NOS (iNOS) [7]. COX is the molecular target for analgesic and anti-inflammatory

* These authors contributed equally to this work

remedies that have been used for hundreds of years. COX exists in two isoforms, the constitutive COX-1 and inducible COX-2 and is produced in abundance by activated macrophages and other cells at the site of inflammation [4]. PGs and NO biosynthesis are involved in inflammation, and iNOS and COX-2 are responsible for the production of large amounts of these pro-inflammatory mediators [22]. CRP is an acute phase protein produced by hepatocytes whose serum elevation is considered as an indicator of chronic inflammation and whose interaction with endothelial cells may be the mechanistic link between CRP and atherosclerosis [17]. Furthermore, CRP induces nuclear factor-κB (NF-κB) activation in circulating monocytes, amplifying the effects of a standard pro-inflammatory stimulus, such as a low dose of lipopolysaccharide (LPS) [14].

NF-κB is activated by various inflammatory stimuli including LPS [15, 28]. Activated NF-κB has been identified in monocyte/macrophages, smooth muscle cells and endothelial cells in human atherosclerotic vessels but not in healthy vessels [3]. NF-κB transcription factors regulate a plethora of cellular pathways and processes including the immune response, inflammation, proliferation, apoptosis and calcium homeostasis [20, 30]. It is especially involved in the expression of CRP, iNOS and COX-2 [5].

Radix puerariae, also called kudzu root, is the root of *Pueraria lobata* (Willd.) Ohwi, which is a perennial leguminous plant native to eastern Asia. Puerarin (7,4'-dihydroxy-8-C-glucosylisoflavone), the chemical structure of which is shown in Figure 1, is the most abundant isoflavone-C-glucoside extracted from *Radix puerariae*. Puerarin has been used for various medicinal purposes in traditional oriental medicine for thousands of years. A number of studies have revealed that puerarin possesses many biological activities, including the degradation of cholesterin [2], antioxidation processes [27], anticarcinogenic processes [31] and the improvement of hyperglycemic disorders [1].

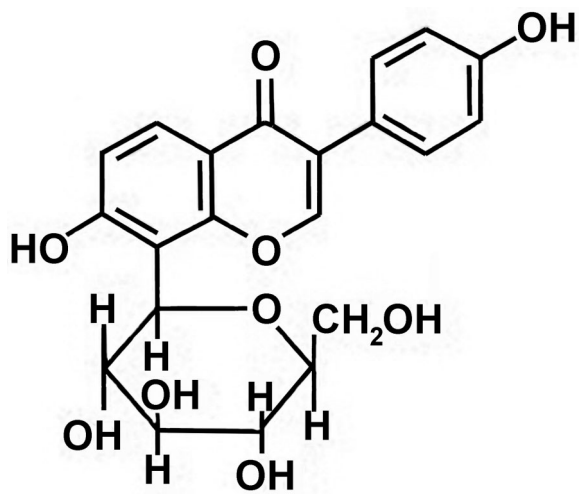

Fig. 1. Chemical structure of puerarin

Flavonoids showed strong inhibitory effects on the expression of COX-2 [24] and iNOS [13] in macrophage cells because of their C2–C3 double bond and 4-oxo functional group of the C-ring, which are important factors for a high level of inhibitory activities. Our preliminary studies suggest that puerarin has an anti-inflammatory property through its inhibitory effect on the expression of CRP protein *via* the suppression of NF-κB activation in LPS-induced peripheral blood mononuclear cells [29]. However, no study to date has documented the potential effects of puerarin on CRP, iNOS and COX-2 expression on this signaling pathway in macrophage cells. Therefore, we investigated the effect of puerarin on CRP, iNOS and COX-2 expression in LPS-induced RAW264.7 macrophage cells. The present study demonstrates that puerarin exhibits its anti-inflammatory activity *via* the suppression of p65NF-κB nuclear translocation and inhibitory-κB (I-κB) phosphorylation.

Materials and Methods

Materials

Puerarin was purchased from Sigma Chemical (St. Louis, MO, USA). RAW264.7 cells and murine macrophages were obtained from the American Type Culture Collection (Rockville, MD, USA).

Cell culture

Cells were maintained at subconfluence in 95% air and 5% CO_2 humidified atmosphere maintained at 37°C. The medium used for routine subculture was Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were counted with a hemocytometer, and the number of viable cells was determined with trypan blue dye exclusion.

MTT assay

An MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-tetrazolium bromide) assay was used to measure the viability of the cells after treatment with puerarin. MTT is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells; an MTT ring is cleaved in active mitochondria, which occurs only in living cells. After the supernatants were removed for nitrite determination, cells were incubated at 37°C with MTT (0.05 mg/ml) for 4 h, and the optical density was measured at 540 nm.

Determination of PGE_2, CRP and nitrite

The culture medium of the control and treated cells was collected, centrifuged and stored at –70°C until further analysis. PGE_2 and CRP proteins secretions in culture supernatants were quantified using an enzyme-linked immunosorbent assay (ELISA) kit obtained from BioSource International (Camarillo, CA), which was used as described by the manufacturer. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction.

Western blot assay

Cells were washed three times with Dulbecco's phosphate buffer saline (DPBS). The washed cells were re-suspended in lysis buffer (10 mM Tris-HCl pH 7.5, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors cocktails) and incubated for 30 min on ice. The lysate was centrifuged at 12,000 × g for 20 min and the supernatant was collected. The protein concentration was determined with the Bio-Rad Protein Assay Reagent (Vancouver, Canada). Protein (80 μg) was separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (8% for iNOS; 10% for COX-2, CRP, IκBα, p-I-κBα, and NF-κB p65) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA). The transblotted membranes were washed twice with TBS containing 0.1% Tween 20 (TBST) and incubated with blocking solution (5% skim milk) for 2 h. The membranes were incubated overnight at 4°C with anti-iNOS, COX-2, CRP, IκBα, p-I-κBα, NF-κB p65 and β-tubulin (lnvitrogen, USA). Blots were washed three times with TBST for 20 min and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (lnvitrogen, USA) for 1 h at room temperature. Blots were again washed three times in TBST and then developed for visualization using the ECL Plus detection kit (Amersham, UK). The intensity of each band was quantitatively determined using Gel-Pro Analyzer Software (Media Cybernetics, USA), and the density ratio represents the relative intensity of each band against those of the controls in each experiment.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed with total RNA. RNA was extracted using TRI REAGENT™ according to the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO). The purity of the RNA preparation was checked by measuring the absorbance ratio at 260/280 nm. The sense and antisense primers for CRP were 5'-AGAATCTGACTTACCCATGGT-3' and 5'-GAGGGAGAAGAATTATGTCTG-3', respectively. The sense and antisense primers for iNOS were 5'-CCCTTCCGAAGTTTCTGGCAGC-3' and 5'-GGC-TGTCAGAGCCTCGTGGCTT-3', respectively. The sense and antisense primers for COX-2 were 5-GGA-GAGACTATCAAGATAGTGATC-3 and 5'-AT- GTCA-GTAGACTTTTACAGCTC-3', respectively. The sense and antisense primers for rat GAPDH mRNA expression (used as a control for total RNA content for each sample) were 5'-TGAAGGTCG- GTGTGAACGGAT-TTGGC-3' and 5'-CATGTAGGC- CATGAGGTCCA CCAC-3', respectively. RT-PCR was performed using the ONE-STEP RT-PCR Pre Mix kit (lnvitrogen, USA), according to the manufacturer's instructions.

Preparation of cytosolic extract

This procedure was carried out by BD Mercury Trans-Factor Kits (BD Biosciences, USA). The treated RAW264.7 cells were collected and rinsed with 20 ml of cold PBS (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.5). Lysis buffer (supplied with a kit) was added to the pellet and was left on ice for 15 min. Next, the cell suspension was centrifuged at 420 × g and 4°C for 5 min, and the supernatant was discarded. Lysis buffer was added to the remaining pellet. The cells were ruptured by rapid strokes of a 27 gauge needle and syringe and centrifuged for 20 min at 4°C at 11,000 × g. The supernatant collected was snap-frozen in liquid nitrogen. Protein content was measured using a BCA assay (Pierce, USA).

Preparation of nuclear extract

The pellet acquired from the cytosolic protein extraction was added with an extraction buffer (which was supplied with the kit). As described in the cytosolic protein extraction procedure, the cell nuclei were disrupted with a 27 gauge needle and syringe and centrifuged at 21,000 × g for 5 min. The nuclear protein in the supernatant was snap-frozen in liquid nitrogen for storage and measured for protein content using the BCA assay (Pierce, USA).

Analysis of p65/DNA binding

DNA binding activity was quantified using the ELISA-based Trans-AMTM NF-κB p65 Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, nuclear extracts (5 μg of protein per well) were incubated in 96-well plates coated with immobilized oligonucleotide containing a NF-κB consensus binding site. NF-κB binding to target oligonucleotides was detected by incubation of the samples with primary antibodies against the p65 subunit provided with the kit. For the quantification of activity, optical densities were measured at 450 nm with a microplate reader.

Data analysis

Results were expressed as the mean ± standard deviation (SD). Differences in mean values between groups were analyzed by a one-way analysis of variance. *Post-hoc* comparisons were carried out by the Student-Newman-Keuls test. Data points were considered statistically significant at $p < 0.05$.

Results

Effect of puerarin on cell viability

The cytotoxicity experiments in this study were performed at 10, 20, 40 and 100 μM concentrations. The puerarin was not cytotoxic at any of the concentrations tested (data not shown).

Effect of puerarin on PGE_2, CRP and NO production

The effects of puerarin on PGE_2, CRP and NO production in LPS-activated RAW264.7 macrophage cells were tested to investigate its anti-inflammatory effects. The amount of nitrite accumulated in the culture medium was estimated using Griess reagent as an index for NO. As shown in Figure 2, The effects of puerarin on PGE_2, CRP and NO production were barely detectable in unstimulated cells but markedly increased after LPS treatment. Further, puerarin inhibited PGE_2, CRP and NO production in a concentration-dependent manner.

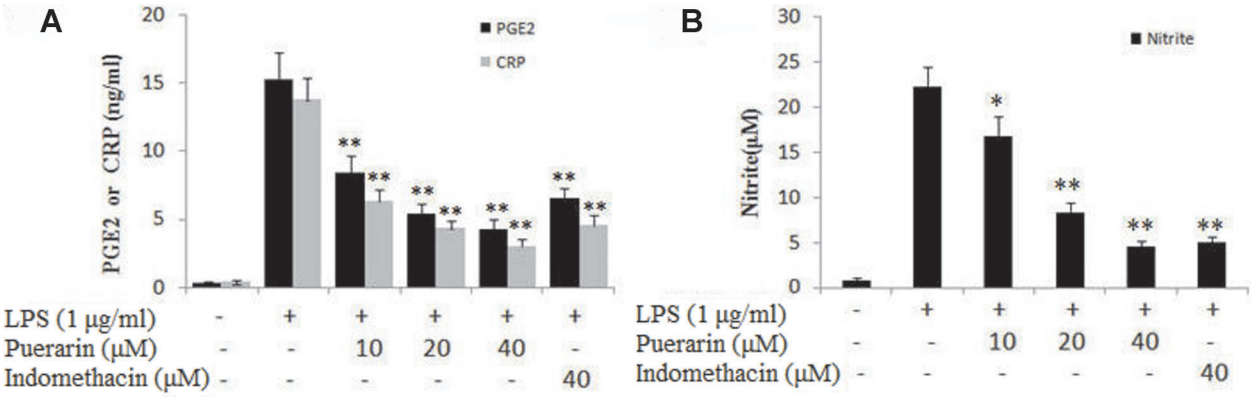

Fig. 2. The LPS-induced inhibition of PGE_2 or CRP (**A**) and NO (**B**) production by puerarin in RAW264.7 macrophage cells. The cells were pretreated with 10, 20, 40 μM of puerarin and 40 μM of indomethacin for 2 h and then incubated with LPS (1 μg/ml) or LPS only for 20 h. The supernatants were harvested and assayed for PGE_2, CRP and nitrite production. Asterisks indicate significant differences from the LPS only incubation (* $p < 0.05$, ** $p < 0.01$)

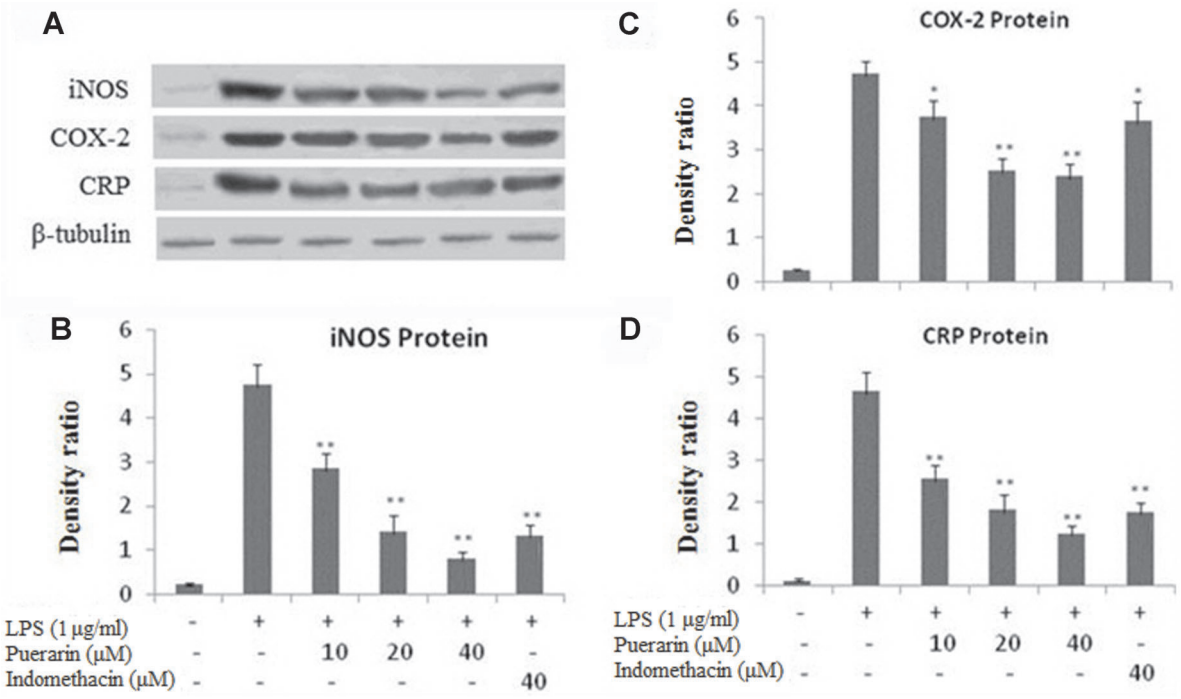

Fig. 3. Inhibition of LPS-induced iNOS (**B**), COX-2 (**C**) and CRP (**D**) protein expression by puerarin in RAW264.7 macrophages. The cells were pretreated with 10, 20, 40 μM of puerarin and 40 μM of indomethacin for 2 h and then incubated with LPS (1 μg/ml) or LPS only for 20 h. Equal amounts of total protein (80 μg/lane) were subjected to 8% (for iNOS) and 10% (for COX-2, CRP and β-tubulin) gels. SDS–PAGE and the expression of iNOS, COX-2, CRP and β-tubulin was detected by western blot analysis (**A**). β-Tubulin was used as an internal control. Asterisks indicate significant differences from the incubation with LPS alone (* $p < 0.05$, ** $p < 0.01$)

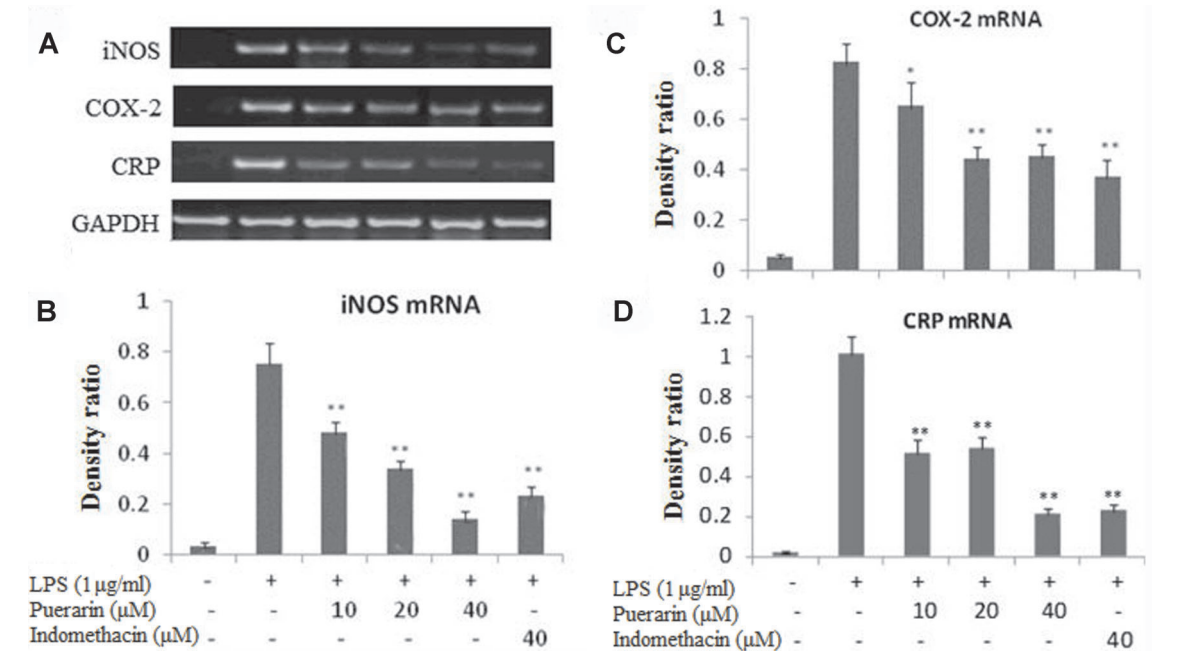

Fig. 4. Effect of puerarin on LPS-induced iNOS (**B**), COX-2 (**C**) and CRP (**D**) mRNA expression in RAW264.7 macrophages. The cells were pretreated with 10, 20, 40 μM of Puerain and 40 μM of indomethacin for 2 h and then incubated with LPS (1 μg/ml) or LPS only for 7 h. Total RNA of lysed cells was prepared for the RT-PCR analysis (**A**). PCR of glyceraldehydes-3-phosphate dehydrogenase, GAPDH, was performed to control for a similar initial cDNA content of sample. Asterisks indicate significant differences from the LPS alone (* $p < 0.05$, ** $p < 0.01$)

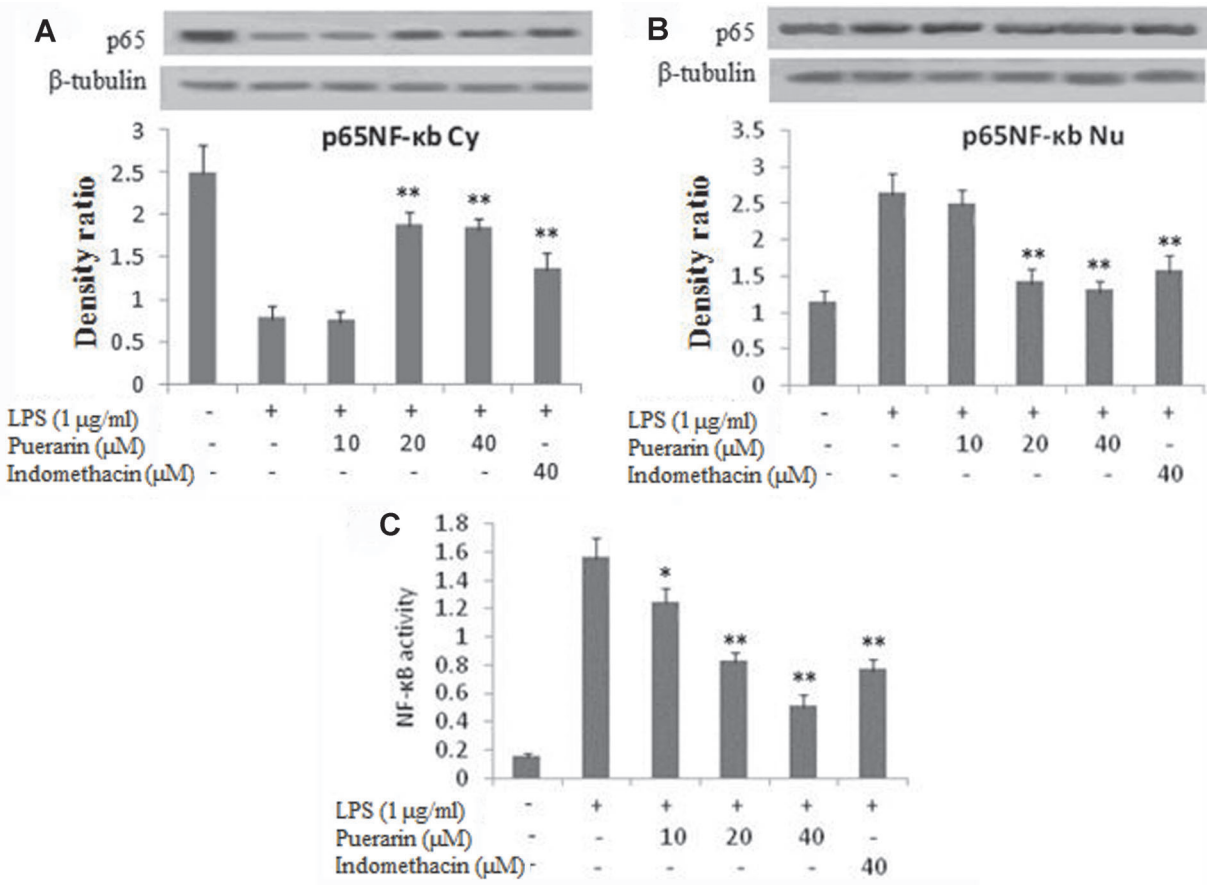

Fig. 5. Effect of puerarin on p65NF-κB expression and NF-κB nuclear protein-DNA binding activity in RAW264.7 macrophages. Cytosolic proteins (**A**) and nuclear proteins (**B**) from cells were pretreated with 10, 20, and 40 μM of puerarin and 40 μM of indomethacin for 2 h and then incubated with LPS (1 μg/ml) or LPS only for 1 h. (**C**) Detection of NF-κB binding activity was performed with the ELISA-based Trans-AM™ NF-κB p65 Kit. Asterisks indicate significant differences from the LPS alone (* $p < 0.05$, ** $p < 0.01$)

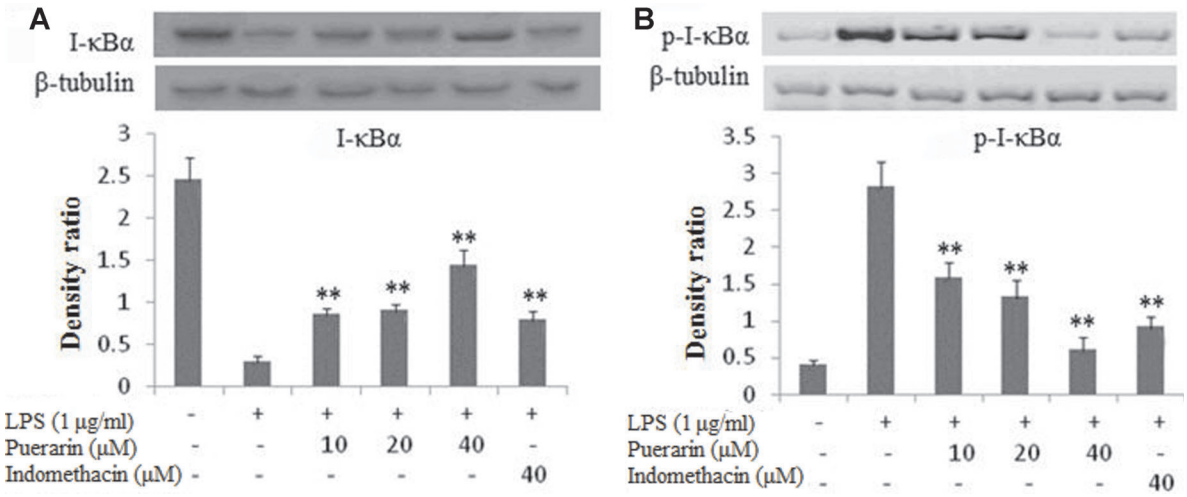

Fig. 6. Effect of puerarin on I-κBα phosphorylation in RAW264.7 macrophages. The cells were pretreated with 10, 20, 40 μM of puerarin and 40 μM of indomethacin for 2 h and then incubated with LPS (1 μg/ml) or LPS only for 1 h. Detection of I-κBα and p-I-κBα expression was determined by western blot analysis. Asterisks indicate significant differences from the LPS alone (** $p < 0.01$)

Effect of puerarin on iNOS, COX-2 and CRP proteins expression

The effects of puerarin on CRP, iNOS and COX-2 protein expression in RAW264.7 cells were examined by western blot analysis. As shown in Figure 3, the CRP, iNOS and COX-2 proteins were barely detectable in unstimulated cells but markedly increased after LPS treatment. In addition, puerarin inhibited expression of CRP, iNOS and COX-2 proteins in a concentration-dependent manner.

Effect of puerarin on iNOS, COX-2 and CRP mRNA expression

We extended our studies to determine whether the expression of CRP, iNOS and COX-2 proteins paralleled those of the corresponding mRNAs. mRNA expression of CRP, iNOS, COX-2 and GAPDH were measured by RT-PCR. As shown in Figure 4, the CRP, iNOS and COX-2 mRNAs were barely detectable in unstimulated cells but markedly increased after LPS treatment. Further, puerarin inhibited expression of CRP, iNOS and COX-2 mRNA in a concentration-dependent manner.

Effect of puerarin on p65NF-κB expression and NF-κB nuclear protein-DNA binding activity

We evaluated the effect of puerarin on the cellular distribution of the p65NF-κB protein after puerarin treatment. Figure 5 shows that puerarin prevented the nuclear translocation of cytosolic p65NF-κB because increasing amounts of p65NF-κB were retained in the cytosol in a dose-dependent manner (Fig. 5A). Correspondingly, decreased amounts were found in the nuclear extract (Fig. 5B). We also determined the effect of puerarin on p65 DNA-binding activity. In the presence of puerarin at 10–40 μM, the DNA-binding activity of NF-κB was suppressed in a dose-dependent manner (Fig. 5C).

Effect of puerarin on I-κBα phosphorylation in RAW264.7 cells

Since the nuclear translocation of p65NF-κB is regulated *via* the phosphorylation of its inhibitor I-κBα, we determined whether puerarin had any effect on the phosphorylation of this molecule. Figure 6A shows the dose-dependent accumulation of I-κBα in LPS-induced cells. Figure 6B confirms that the accumulation of I-κBα is due to the dose-dependent inhibition of puerarin by the phosphorylation and subsequent degradation of I-κBα.

Discussion

The activation of macrophages plays an important role in the inflammatory process [9]. Increases in iNOS, COX-2 and CRP gene expression are associated with inflammatory responses [5]. Continuous production of these molecules in chronic inflammation has been linked to the development of autoimmune disorders, coronary artery disease and cancer [16, 19, 23]. The interaction between CRP and endothelial cells has been the focus of many studies. In previous studies, CRP was found to induce NF-κB activation in rat vascular smooth muscle cells [8], bovine aortic endothelial cells [12] and human saphenous vein endothelial cells [26]. In this study, the results indicated that puerarin inhibits the expression of the protein and mRNA levels of iNOS, COX-2 and CRP in LPS-induced RAW264.7 cells.

During macrophage inactivation, NF-κB is maintained in the cytosol and bound by inhibitory-κB proteins (I-κB). Only when NF-κB has been released will it translocate to the nucleus and bind specific κB sequences in the regulatory regions of target genes [30]. When a macrophage is activated, the nuclear translocation of NF-κB starts with the phosphorylation of NF-κB kinase (IKK) inhibitor by NF-κB-inducing kinase (NIK) [10], followed by the rapid phosphorylation of I-κB by IKK and the degradation of phosphorylated I-κB by the roteosome complex [11]. The phosphorylation of I-κB is generally regarded as the rate-limiting step in the release of NF-κB [6, 30]. The p50/p65 heterodimer is the most common dimer found in the NF-κB signaling pathway [25].Thus, the ability of puerarin to inhibit the phosphorylation and degradation of I-κBα will cause the accumulation of both I-κBα and p65NF-κB in the cytosol, thereby reducing the amount of p65NF-κB that can enter the nucleus. In our study, puerarin inhibited the phosphorylation of I-κB, blocked the I-κB production and furthermore suppressed p65 NF-κB translocation to the nucleus and modulated its binding activity.

In conclusion, we have shown that puerarin inhibits CRP, iNOS and COX-2 expression in RAW264.7 macrophage cells. The mechanism of this effect, at least in part, may involve the inhibition of the NF-κB pathway. Puerarin shows potential as an anti-inflammatory agent and may be used in the future as a novel agent for the chemoprevention of cancer and/or inflammatory diseases, such as atherosclerosis.

Acknowledgment:
This study was supported by An Open Issue Foundation of Jiangsu Provincial People's Hospital (KF200937).

References:

1. Choi J, Shin MH, Park KY, Lee KT, Jung HJ, Lee MS, Park HJ: Effect of kaikasaponin III obtained from Pueraria thunbergiana flowers on serum and hepatic lipid peroxides and tissue factor activity in the streptozotocin-induced diabetic rat. J Med Food, 2004, 7, 31–37.
2. Chung MJ, Sung NJ, Park CS, Kweon DK, Mantovani A, Moon TW, Lee SJ, Park KH: Antioxidative and hypocholesterolemic activities of water-soluble puerarin glycosides in HepG2 cells and in C57 BL/6J mice. Eur J Pharmacol, 2008, 578, 159–170.
3. Collins T, Cybulsky MI: NF-κB: pivotal mediator or innocent bystander in atherogenesis? J Clin Invest, 2001, 107, 255–264.
4. Cuccurullo C, Fazia ML, Mezzetti A, Cipollone F: COX-2 expression in atherosclerosis: the good, the bad or the ugly? Curr Med Chem, 2007, 14, 1595–1605.
5. Garcia-Mediavilla V, Crespo I, Collado PS, Esteller A, Sanchez-Campos S, Tunon MJ, Gonzalez-Gallego J: The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. Eur J Pharmacol, 2007, 557, 221–229.
6. Griscavage JM, Wilk S, Ignarro LJ: Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-κB. Proc Natl Acad Sci USA, 1996, 93, 3308–3312.
7. Hao XP, Pretlow TG, Rao JS, Pretlow TP: Inducible nitric oxide synthase (iNOS) is expressed similarly in multiple aberrant crypt foci and colorectal tumors from the same patients. Cancer Res, 2001, 61, 419–422.
8. Hattori Y, Matsumura M, Kasai K: Vascular smooth muscle cell activation by C-reactive protein. Cardiovasc Res, 2003, 58, 186–195.
9. Hilgendorff A, Muth H, Parviz B, Staubitz A, Haberbosch W, Tillmanns H, Hölschermann H: Statins differ in their ability to block NF-kappaB activation in human blood monocytes. Int J Clin Pharmacol Ther, 2003, 41, 397–401.
10. Jiang X, Takahashi N, Ando K, Otsuka T, Tetsuka T, Okamoto T: NF-κB p65 transactivation domain is involved in the NF-κB-inducing kinase pathway. Biochem Biophys Res Commun, 2003, 301, 583–590.
11. Jijon H, Allard B, Jobin C: NF-κB inducing kinase activates NF-κB transcriptional activity independently of IκB kinase γ through a p38 MAPK-dependent RelA phosphorylation pathway. Cell Signal, 2004, 16, 1023–1032.
12. Kawanami D, Maemura K, Takeda N, Harada T, Nojiri T, Saito T, Manabe I et al.: C-reactive protein induces VCAM-1 gene expression through NF-κB activation in vascular endothelial cells. Atherosclerosis, 2006, 185, 39–46.
13. Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP: Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem Pharmacol, 1999, 58, 759–765.
14. Liuzzo G, Santamaria M, Biasucci LM, Narducci M, Colafrancesco V, Porto A, Brugaletta S et al.: Persistent activation of nuclear factor kappa-B signaling pathway in patients with unstable angina and elevated levels of C-reactive protein evidence for a direct proinflammatory effect of azide and lipopolysaccharide-free C-reactive protein on human monocytes via nuclear factor kappa-B activation. J Am Coll Cardiol, 2007, 49, 185–194.
15. Łabuzek K, Liber S, Gabryel B, Okopień B: Metformin has adenosine-monophosphate activated protein kinase (AMPK)-independent effects on LPS-stimulated rat primary microglial cultures. Pharmacol Rep, 2010, 62, 827–848.
16. Motilva V, Alarcon de la Lastra C, Bruseghini L, Manuel Herrerias J, Sanchez-Fidalgo S: COX expression and PGE_2 and PGD_2 production in experimental acute and chronic gastric lesions. Int Immunopharmacol, 2005, 5, 369–379.
17. Nabata A, Kuroki M, Ueba H, Hashimoto S, Umemoto T, Wada H, Yasu T et al.: C-reactive protein induces endothelial cell apoptosis and matrix metalloproteinase-9 production in human mononuclear cells: Implications for the destabilization of atherosclerotic plaque. Atherosclerosis, 2008, 196, 129–135.
18. Ohshima H, Tazawa H, Sylla BS, Sawa T: Prevention of human cancer by modulation of chronic inflammatory processes. Mutat Res, 2005, 591, 110–122.
19. Pan MH, Lin-Shiau SY, Lin JK: Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IκB kinase and NFκB activation in macrophages. Biochem Pharmacol, 2000, 60, 1665–1676.
20. Patel S, Santani D: Role of NF-κB in the pathogenesis of diabetes and its associated complications. Pharmacol Rep, 2009, 61, 595–603.
21. Prestes-Carneiro LE, Shio MT, Fernandes PD, Jancar S: Cross-regulation of iNOS and COX-2 by its products in murine macrophages under stress conditions. Cell Physiol Biochem, 2007, 20, 283–292.
22. Raso GM, Meli R, Di Carlo G, Pacilio M, Di Carlo R: Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. Life Sci, 2001, 68, 921–931.
23. Salerno L, Sorrenti V, Di Giacomo C, Romeo G, Siracusa MA: Progress in the development of selective nitric

oxide synthase (NOS) inhibitors. Curr Pharm Des, 2002, 8, 177–200.
24. Takano-Ishikawa Y, Goto M, Yamaki K: Structure-activity relations of inhibitory effects of various flavonoids on lipopolysaccharide-induced prostaglandin E_2 production in rat peritoneal macrophages: comparison between subclasses of flavonoids. Phytomedicine, 2006, 13, 310–317.
25. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S: Rel/NF-κB/I κB family: intimate tales of association and dissociation. Genes Dev, 1995, 9, 2723–2735.
26. Verma S, Badiwala MV, Weisel RD, Li SH, Wang CH, Fedak PW, Li RK et al.: C-reactive protein activates the nuclear factor-κB signal transduction pathway in saphenous vein endothelial cells: implications for atherosclerosis and restenosis. J Thorac Cardiovasc Surg, 2003, 126, 1886–1891.
27. Wu L, Qiao H, Li Y, Li L: Protective roles of puerarin and Danshensu on acute ischemic myocardial injury in rats. Phytomedicine, 2007, 14, 652–658.
28. Xanthoulea S, Curfs DM, Hofker MH, de Winther MP: Nuclear factor kappa B signaling in macrophage function and atherogenesis. Curr Opin Lipidol, 2005, 16, 536–542.
29. Yang X, Hu W, Zhang Q, Wang Y, Sun L: Puerarin inhibits C-reactive protein expression via suppression of nuclear factor kappaB activation in lipopolysaccharide-induced peripheral blood mononuclear cells of patients with stable angina pectoris. Basic Clin Pharmacol Toxicol, 2010, 107, 637–642.
30. Yates LL, Górecki DC: The nuclear factor-kappaB (NF-κB): from a versatile transcription factor to a ubiquitous therapeutic target. Acta Biochim Pol, 2006, 53, 651–662.
31. Yu Z, Li W: Induction of apoptosis by puerarin in colon cancer HT-29 cells. Cancer Lett, 2006, 238, 53–60.

Received: August 15, 2010; **in the revised form:** October 17, 2010; **accepted:** November 18, 2010.