Thesis for Ph. D. Degree

February, 2009

Role of Pin1 in the Induction of Proinflammatory Mediatorsand the studies on the Anti-inflammatory effects of Phytochemicals

Chosun University Graduate School

College of Pharmacy

By

Yuba Raj Pokharel

Thesis Submitted to the faculty of

Chosun University in fulfillment of the

Requirements for the degree

Of

Doctor of Philosophy

Under the Supervision of Professor

Keon Wook Kang

February, 2009

Chosun University Graduate School

College of Pharmacy

By

Yuba Raj Pokharel

염증매개물의 유도과정에서 Pin1 의 역할 및 천연물의 항염증효과연구

Role of Pin1 in the Induction of Proinflammatory Mediators and the studies on the Anti-inflammatory effects of Phytochemicals

2009년 12월
조선대학교 대학원
약 학 과
유바 라쥐 포카렐

Role of Pin1 in the Induction of Proinflammatory Mediators and the studies on the Anti-inflammatory effects of Phytochemicals

지도교수　강 건 욱

이 논문을 약물학 박사학위신청 논문으로 제출함

2009년 12월

조선대학교 대학원

약학과

This thesis is examined and approved for the Ph.D. degree

Chairman Chosun Univ. **Dr. Jeong Hye Gwang (印)**

Member Chosun Univ. **Dr. Oh Won Keon (印)**

Member Chosun Univ. **Dr. Choi Hong Seok (印)**

Member Chonnam Nat'l Univ. **Dr. Lee Kwang Yeol (印)**

Member Chosun Univ. **Dr. Kang Keon Wook(印)**

2009년 12월

Chosun University Graduate school

CONTENTS

Part two

ABSTRACT

Role of Pin1 in the Induction of Proinflammatory Mediators and the studies on the Anti-inflammatory effects of Phytochemicals

Pokharel Yuba Raj
Advisor: Prof. Kang Keon-Wook Ph.D
Department of Pharmacy,
Graduate School of Chosun University

Rheumatoid arthritis (RA) is an autoimmune disease, characterized by chronic inflammation in joints and subsequent destructions of cartilage and bone. Inflammatory mediators such as prostaglandins and proinflammatory cytokines are believed to be associated with RA progress. Pin1, a peptidyl prolyl isomerase, plays important pathophysiological roles in several diseases including cancer and neurodegeneration. We found that Both Pin1 and cyclooxygenase-2 (COX-2) were highly expressed in ankle tissues of Type II collagen-induced RA mice. In the Pin1-overexpressed HTB-94 cells and -primary cultured human chondrocytes, the basal expression of proinflammatory proteins (COX-2, inducible nitric oxide synthase, tumor necrosis factor-α and interleukin-1β) was increased compared to the GFP-overexpressed cells. Site-specific mutation analyses revealed that Pin1-mediated transcriptional activation of *COX-2* gene was coordinately regulated by nuclear

factor-κB (NF-κB), cyclic AMP response element binding protein (CREB) and CCAAT-enhancer binding protein. Gel shift, reporter gene and Western blot analyses confirmed that NF-κB, CREB and C/EBP were consistently activated in the Pin1-overexpressed chondrocyte cell line. Treatment of RA mice with juglone, a chemical inhibitor of Pin1, significantly reduced the RA progress and COX-2 expression in the ankle tissues. Moreover, the basal COX-2 expression in primary cultured chondrocytes from RA patients was diminished by juglone in a concentration-dependent manner. These results demonstrate that Pin1 induction during RA progress stimulates proinflammatory protein expression by activating NF-κB, CREB, C/EBP and AP-1, and suggest that Pin1 is a potential therapeutic target of RA.

The improper productions of NO and prostaglandins following the inductions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are involved in the pathogenesis of chronic inflammation. *Selaginella tamariscina* is used as an oriental medicine for its anti-inflammatory effects. Here, we isolated taiwaniaflavone and 2', 8”-biapigenin from *S. tamariscina* and investigated whether taiwaniaflavone, and 2’, 8”-biapigenin affect the

induction of iNOS and COX-2 in RAW264.7 macrophages stimulated with lipopolysaccharide (LPS). We found that taiwaniaflavone blocks the transactivations of *iNOS* and *COX-2* genes by blocking the nuclear translocation of p65 and subsequent nuclear factor-κB inactivation. It is known that NF-κB activation is controlled by the phosphorylation and subsequent degradation of I-κB, and in the present study, we found that the phosphorylation and degradation of I-κB ˛ were also inhibited by taiwaniaflavone. Our findings indicate that taiwaniaflavone and 2', 8”-biapigenin may provide a developmental basis for an agent against inflammatory diseases.

We recently isolated a novel lignan, 4-hydroxykobusin from *Geranium thunbergii* (Liu et al., 2006). Here, we studied its effect on the expression of *inducible nitric oxide synthase* (*iNOS*) gene in RAW264.7 cells. 4-hydroxykobusin inhibited NO production in a concentration-dependent manner and blocked the LPS-induced expression of inducible nitric oxide synthase (iNOS). To identify the mechanistic basis for its inhibition of iNOS induction, we examined the effect of 4-hydroxykobusin on the transactivation of iNOS gene by luciferase reporter activity using –1.59 kb flanking region. The

lignan suppressed the reporter gene activity and the LPS-induced reporter activations of NF-κB and AP-1 were also significantly blocked by 4-hydroxykobusin. These findings suggest that the inhibition of LPS-induced NO formation by 4-hydroxykobusin is due to its inhibition of NF-κB and AP-1 activation.

Abbreviations

Activator Protein-1 (AP-1)

cAMP response element binding protein(CREB)

CCCAAT/ enhancer binding protein (C/EBP)

CCCAAT/ enhancer binding protein α (C/EBPα)

CCCAAT/ enhancer binding protein β (C/EBPβ)

Cyclooxigenase-1 (COX-1)

Cyclooxigenase-2 (COX-2)

Collagen type II (CII)

Extracellular signalregulated kinase (ERK)

Extra cellular matrix (ECM)

Fetal bovine serum (FBS)

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Green Fluorescence Protein (GFP)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Interferon γ (IFN γ)

Interleukin1 β (IL-1 β)

Inhibitor-κB (I- κB)

Inducible nitricoxide synthase (iNOS)

Jun NH2-terminal kinase **(**JNK)

Lipopolysaccharide (LPS)

Mitogen -activated protein kinase (MAPK)

Mitogen- activated protein kinase kinase (MKK)

Matrix metalloproteinase (MMP)

Messenger riboneuclic acid (mRNA)

Nuclear factor-κB (NF- κB)

Nitric oxide (NO)

Nonsteroidal Anti-inflammatory drugs (NSAID)

Osteoarthritis (OA)

Phosphate buffered saline (PBS)

Prostaglandin (PG)

Prostaglandin E synthase (PGES)

Prostaglandin E 2 (PGE2)

Phosphatidylinositol 3- kinase (PI3K)

Peptidyl-prolyl cis/trans isomerase1 (Pin1)

Protein kinase A (PKA)

Protein kinase C (PKC)

Rheumatoid arthiritis (RA)

Reactive oxygen species (ROS)

Reverse transcription-polymerase chain reaction (RT-PCR)

Sodium dodecylsulphate (SDS)

Small interference riboneuclic acid (siRNA)

Signal Transducer and Activator of Transcription 3 (STAT3)

Tumor growth factor (TGF)

Tumer necrosis factor α (TNF α)

Tosyl phenylalanyl chloromethyl ketone (TPCK)

List of Figures and Table

1. Introduction

1.1 Definition of Inflammation ,RA, Etiology and Epidemiology of RA

1. 1. 1. Inflammation

Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissues. In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, inflammation which runs unchecked can aslo lead to a host of diseases, such as hay fever, artherossclerosis, and rheumatoid arthritis. Inflammation can be classified as either acute or chronic. Acute inflammation is the intial response of the body of harmful stimuli and is achieved by the increased movement of plasma and leukocyte from the blood intothe injured tissues. A cascade of biochemical events propagates and matures inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation leads to a

progressive shift in the type of ccells which are present at the site of clls which are present at the site of inflammation and is charcterised by simultaneousdestruction and healing of the tissue from inflammatory process (Williams & Wilkins, 1990).

1. 1. 2. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder that causes the immune system to attack the joints, where it causes inflammation (arthritis) and destruction of catilage and bone. It can also damage some organs, such as the lungs and skin. It can be a disabling and painful condition, which can lead to substantial loss of functioning and mobility. It is diagnosed with blood tests (especially a test called rheumatoid factor) and X-rays (Majithia et al, 2007).

The etiology of rheumatoid arthritis is not fully understood but environmental and genetic factors play a crucial role in RA. A triggering event, possibly autoimmune or infectious, initiates joint inflammation. Complex interactions among multiple immune cell types and their cytokines, proteinases, and growth factors mediate joint destruction and systemic complications. (Firestein et al, 2005).

The incidence of RA is in the region of 3 cases per 10,000 population per annum. Onset is uncommon under the age of 15 and from then on the incidence rises with age until the age of 80. It is 4 times more common in smokers than non-smokers. Some Native American groups have higher prevalence rates (5-6%) and people from the Caribbean region have lower prevalence rates (Symmons et al., 2002; Alamanos et al., 2006).

Rheumatoid arthritis affects women three times more often than men, and it can first develop at any age. The risk of first developing the disease (the disease incidence) appears to be greatest for women between 40 and 50 years of age, and for men somewhat later. RA is a chronic disease, and although rarely, a spontaneous remission may occur, the natural course is almost invariably one of persistent symptoms, waxing and waning in intensity, and a progressive deterioration of joint structures leading to deformations and disability.

1. 2.. Molecular and cellular pathogenesis of RA

Rheumatoid arthritis (RA) is an autoimmune disease, which is characterized by chronic inflammation in joints through leukocytes

sequestration and subsequent destructions of cartilage and bone. Although the exact pathological process has not been clearly clarified, inflammatory mediators including prostaglandins (PGs) and proinflammatory cytokines are believed to be associated with RA progress (Feldmann et al., 1996). A key enzyme to control PGs production in RA is cyclooxygenases-2 (COX-2). The increased COX-2 expression has been frequently found in either RA patients or animal arthritis models (Siegle et al., 1998; Anderson et al., 1996). The overwhelmed production of PGs by cytokines-inducible COX-2 is closely associated with angiogenesis and inflammation of the synovial membrane in RA (Myers et al., 2000). In fact, proinflammatory cytokines such as TNF-α and IL-1β induce COX-2 in RA models (Bidgood et al., 2000; Martel-Pelletier et al., 2003).

Pin1, a peptidyl prolyl isomerase, was originally discovered in a screen for elucidating mitosis-associated molecules (Lu et al., 1996). Pin1 specifically recognizes phosphorylated serine or threonine immediately preceding proline (pSer/Thr-Pro) and then isomerizes the peptide bond (Bayer et al., 2003; Lu et al., 2004). Pin1-dependent isomerization is important for its target proteins activities, because various protein kinases or phosphatases recognize their substrates in a

conformation-dependent manner (Weiwad et al., 2000; Zhou et al., 2000). Most researches to find pathophysiological roles of Pin1 have been focused on cancer, since Pin1 overexpression was frequently observed in several types of cancer tissues (Bao et al., 2004). Recent studies have also revealed that Pin1 plays a protective role in the development of neurodegenerative disease (Balastik et al., 2007) and may potentiate the outcomes of hepatitis B virus infection via physical interaction with hepatitis B virus X protein (Pang et al., 2007). The functions of Pin1 in immune system have been studied by Malter group. They found that Pin1 regulates the mRNA stabilities of transforming growth factor-β (TGF-β) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in eosinophils and T lymphocytes (Shen et al., 2005; 2008; Esnault et al., 2006). Although, diverse functions of Pin1 have been elucidated as aforementioned, the pathological role of Pin1 in RA has not been studied.

Type II collagen (CII)-induced arthritis in DBA1/J mice has been proven to be a useful model of RA. Humoral and cell immunity characteristics of the mice are very similar to those of RA patients (Holmdahl et al., 2000). In the present study, we found that Pin1 and COX-2 were highly induced in chondrocytes, lymphocytes and

fibroblasts of arthritic joints obtained from CII-injected DBA/1J mice. To clarify the pathological role of Pin1 overexpression, we established HTB-94 cells and primary cultured human chondrocytes stably overexpressing Pin1. The basal expressions of COX-2, inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were enhanced in both the Pin1-overexpressing cell types and Pin1-dependent activations of nuclear factor-κB (NF-κB), cAMP response element binding protein (CREB) and CCAAT-enhancer binding protein (C/EBP) are involved in the COX-2 induction.

1. 3. **Role of iNOS and COX-2 in inflammation**

Nitric oxide (NO) plays beneficial and determental roles during inflammation. NO produced by constitutive NOS forms (cNOS, or NOS type III and I) is essential for maintaining cellular function (Porsti et al., 1995), where as NO produced by inducible NOSs (iNOS, NOS type II is an important mediator of acute and chronic inflammation (Kubes et al., 2000), and contributes to the pathogenesis of organ failure in circulatory shock (Southan et al., 1996).

Cyclooxygenase (COX) is a rate-limiting enzyme in the conversion of arachidonic acid into prostaglandins and thromboxanes. The enzyme plays several important roles in maintaining physiological

homeostasis, such as mucosa secretion and smooth muscle contraction, and in regulating pathological conditions, such as allergic diseases and rheumatoid arthritis (Goetzl et al., 1995). There are two isoforms of cyclooxygenase, i.e., COX-1 and COX-2 (Hla et al., 1992). COX-1 functions as a housekeeping gene and is constitutively expressed in most human tissues, whereas COX-2 is an inducible form that is induced by oncogenes, growth factors, cytokines, endotoxin or phorbol esters (Arias-Negrete et al., 1995). Overexpression of COX-2 has been related to chronic inflammation, angiogenesis and carcinogenesis (Tsuji et al., 2001).

Recently, it was suggested that chronic inflammation is associated with carcinogenesis (Oshima et al., 2003; Farrow et al., 2002). Chronic inflammation leads to the induction of specific enzymes in affected tissues and cells. In particular, inducible nitric oxide synthase (iNOS) and cyclooxigenase-2 are responsible for the exaggerated production of NO and prostaglandins, respectively, believed to be involved in the pathogenesis of cancer (Lala et al., 2001; Zha et al., 2004) COX-2 participates during the gastric tumurogenesis(Van Rees et al., 2002). It has been reported that there is strong positive relationship between the presence of iNOS and the

frequency of mutation in colon tumor tissues (Ambs et al., 1999). Hence, the overproduction of prostaglandins and NO may act as both an endogenous initiator and as a promoter of carcinogenesis and specific inhibitors of COX-2 or iNOS might have applications as chemopreventive agents in human cancer.

1. 4. Transcriptional Regulation of iNOS, COX-2 and role of Pin1

Pin1 may act as a novel pathological mediator to stimulate the transcription of proinflammatory proteins in RA tissues. The cis-acting elements in the 50-flanking promoter region of the COX-2 gene contain a TATA box and multiple transcription factor binding sites for nuclear factor-kB (NF-κB), specific protein-1, Myb, CCAAT/enhancer-binding protein (C/EBP), and cAMP response element binding protein (CREB) (Kosaka et al., 1994). Among these transcription factors, C/EBP, CREB, and NF-κB play important roles in the induction of COX-2 (Kim et al., 1998; Tang et al., 2001; Wu et al., 2003).

The promoter regions of the *iNOS* and *COX-2* genes contain NF-κB binding sites (Schmedtje et al., 1997; Xie et al., 1993), and NF-κB is known to be an essential transcription regulator of these two genes (Diaz-Guerra et al., 1996; Lee et al., 2003). The stimulation of cells by diverse inflammatory insults results in the phosphorylation of

the I-κB/NF-κB complex and the subsequent degradation of I-κB proteins. The degradation of I-κB causes the dissociation of the NF-κB complex from the I-κB protein, which allows free NF-κB to enter the nucleus. Nuclear NF-κB, which is a member of a transcription complex, in turn regulates the expression of the *iNOS* and *COX-2* genes. The phosphorylation of I-κB bound to NF-κB is believed to be mediated by I- κB kinase at two conserved serines within the N-terminal domain of I-κB (Karin et al .,2000) and the I-κB kinase complex can be activated by a variety of upstream kinases (Huang et al., 2003; Trushin et al., 2003).

Recently there is a report that peptidyl-proline isomerase Protein Never in Mitosis Gene A Interacting-1 (Pin1) activity of several transcription factors that can induce the inducible nitric oxide (NO) synthase (iNOS) as well as Pin1 can also regulate mRNA and protein turnover (Liyu et al., 2008). Pin1 enhances Stat3- mediated epithelial-mesenchymal transition in breast cancer cells induced by oncostatin M. Stat3 is an important cytoplasmic transcription factors for cytokine signaling (Lufei et al., 2007). With the stimulation of cytokines Pin1 binds to the pThr254- promotif in p65 and inhibit p65 binding to I-κB alpha, increased the nuclear accumulation and protein stability of p65

and increased the NF-κB (Ryo et al., 2003).

1. 5. Therapeutic use of Phytochemicals in Inflammation.

Chemoprevention is considered to be one of the most promising strategies for the prevention of human cancers. It is defined as the use of either natural or synthetic compounds to block or retard the carcinogenic process, and many natural candidates including epigallocatechin, genistein and sulforaphane have been evaluated in terms of malignancy prevention (Moyers et al., 2004; Sarkar et al., 2003; Chung et al., 2000).

The leaves of *Selaginella tamariscina*, which are used in oriental medicine, have been reported to lower blood glucose levels and facilitate the repair of pancreatic islet B cells injured by alloxan (Miao et al., 1996). Crude extracts of *S. tamariscina* also reduced the productions of proinflammatory cytokines, e.g., interleukin-1β and tumor necrosis factor-α in human mesangial cells (Kuo et al., 1998). In the present study, we isolated a bioflavonoid, taiwaniaflavone from the ethylacetate fraction of *Selaginella tamariscina*. Taiwaniaflavone has been isolated from several plants (e.g. *Taiwania cryptomerioides* Hayata) (Kamil et al., 1981; Chien et al., 2004), but its pharmacological activities have not been studied. Lee et al., 1999;

reported that the water-extracted fraction of *Selaginella tamariscina* (Selaginellaceae) efficiently increased p53 gene expression and induced G1 arrest, suggesting that *S. tamariscina* is a candidate chemopreventive. Crude extracts of *S. tamariscina* also reduced the production of proinflammatory cytokines, interleukin-1β and tumor necrosis factor- α in human mesangial cells (Kuo et al., 1998). As a part of our program to screen for potential cancer chemopreventive compounds from medicinal plants, we isolated 2', 8''-biapigenin from *S. tamariscina* (Fig. 1). The biological activity of 2', 8''-biapigenin has not been studied. In the present study, we investigated the modulatory effects of the bi-flavonoid taiwaniaflavone and 2', 8''-biapigenin on the expressions and activities of iNOS and COX-2 induced by lipopolysaccharide (LPS) in RAW264.7 macrophage cells.

Geranium thunbergii, which is widely used as an anti-diarrhetic agent in East Asia, (Okuda et al., 1975) has been reported to have anti-mutagenicity, anti-inflammation and anti-oxidative effects (Hiramatsu et al.,2004; Ushio et al., 1991; Xiufen et al., 2004). One of representative tannin in Geraniaceae, geraniin shows diverse effects including anti-bacterial, anti-fungal and anti-hypertension (Cheng et al., 1994; Gohar et al., 2003). We recently isolated three

lignans (kobusin, 7, 7’-dihydroxybursehernin and 4-hydroxykobusin) from *Geranium thunbergii* (Liu et al., 2006). Among them, 4-hydroxykobusin has been identified as a new furofuran lignan and is effective to inhibit interleukin-6 production in MG-63, a human osteosarcoma cell line (Liu et al., 2006).

2. Study Aim.

1. To determine the role of Pin induction in the expression of proinflammatory proteins in RA model.
2. Screening of useful phytochemicals for the therapeutic use in chronic inflammatory diseases and to determine their pharmacological mechanisms.

3. Materials and Methods

3.1. Materials.

5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium were supplied by Life Technologies (Gaithersburg, MD). Anti- Pin1, COX-2, C/EBP , C/EBP , CREB, c-Jun, c-Fos, JunD, Fra1 and p65 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Anti-murine iNOS polyclonal antibody from Transduction Laboratories (Lexington, KY); anti- I-κB and anti-phospho-I-κB kinase (IKK)/IKK antibodies from Cell Signaling Technology (Beverly, MA). Phosphorylated form-specific or total form recognizing antibodies against extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated donkey anti-rabbit and alkaline phosphatase-conjugated donkey anti-mouse IgGs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The reagents used for molecular studies were primarily obtained from Sigma (St. Louis, MO). siRNA targeting human c-Jun and control siRNA were purchased from Ambion (Austin, TX).

3. 2. CII-induced arthritis and juglone treatment.

The institutional animal care and utilization committee of Chosun University approved all the animal procedures used in this study. Male DBA/1J mice (Joong-Ang Experimental Animals Co., Seoul, Korea), age 8 weeks, were used. Bovine CII was dissolved in 0.1 M acetic acid overnight at 4 °C. This was emulsified in an equal volume of complete Freund's adjuvant (Sigma). The mice were immunized intradermally at the base of the tail with 100 μl emulsion containing 150g CII. On day 21, mice were boosted intradermally with 100g CII dissolved in PBS and monitored arthritis development for 10 days. Juglone was dissolved in solubilization solvent (PEG400, Tween 80, ethanol and sterile water) and intraperitoneally injected from day 22 every other days (4 times injection).

3. 3. Assessment of arthritis.

Mice were sacrificed on day 10 after second CII booster. The left hind limbs including paws and ankles were dissected, fixed immediately for 12 h in 10% neutralizing formaldehyde, decalcified in Calci-Clear Rapid^{TM} (National Diagnostics, Atlanta, GA) for 12h, and embedded in paraffin. Tissue sections (4 μm) were mounted on common slides for staining with hematoxylin and eosin. A certified pathologist scored samples in a blinded fashion. The data were

expressed as mean chronic inflammation, fibrosis, articular cartilage damage, synovialis proliferation, and bone damage and ankylosis scores. All scores were semiquantitatively indexed based on a scale of 0-3 (Leng et al., 2008).

3. 4. Immunohistochemistry.

A universal immunoenzyme polymer method was used for immunostaining. 4 μm sections were cut from formalin-fixed, paraffin-embedded tissue blocks, mounted on polylysine-coated slides, dewaxed in xylene, and rehydrated through a graded series of ethanol. After deparaffinization, antigen retrieval treatment was performed at 121 °C for 15 min in 10 mM sodium citrate buffer (pH 6.0), and was then treated with 3% hydrogen peroxide in methanol solution for 20 min in order to quench endogenous peroxidase activity. To block intrinsic avidin–biotin capabilities, the tissue slides were treated with avidin–biotin blocking kit reagents (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 15 min. Anti-Pin1 and anti-COX-2 antibodies were used as the primary antibodies. The final products were visualized using the 3-3'diaminobenzidine tetrahydrochloride (DAB) detection system (DakoCytomation, Glostrup, Denmark). All experiments were performed in duplicate.

3. 5. Cell culture:

HTB-94 cells and primary cultured human chondrocytes (passage 6) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and Dr. Lee (Chonnam National University, Gwangju, South Korea), respectively. Both the cell types were cultured at 37°C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 g/ml streptomycin. For all experiments, cells were grown to 80-90% confluency and subjected to no more than 15 cell-passages. Raw264.7 cells and J774.A1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and Korean Cell Line Bank (KCLB, Seoul, Korea), respectively. Both the cells were cultured at 37°C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100μg/ml streptomycin. For all experiments, cells were grown to 80%-90% confluency and subjected to no more than 20 cell passages.

3. 6. MTT cell viability assay

Viable adherent cells were stained with MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] (2 mg/ml)

for 4 h. Media were then removed and the formazan crystals produced were dissolved by adding 200 μl of dimethylsulfoxide. Absorbance was assayed at 540 nm and cell viabilities were expressed as ratios versus untreated control cells.

3. 7. Measurement of nitrite

RAW264.7 cells ($5x10^5$ cells) were preincubated at 37 °C for 12 h in serum-free medium and NO production was monitored by measuring nitrite levels in culture media using Griess reagent (1 % sulfanilamide, 0.1 % *N*-1-naphthylenediamine dihydrochloride, and 2.5 % phosphoric acid). Absorbance was measured at 540 nm after incubating for 10 min.

3. 8. Construction of Pin1 retroviral plasmid and infections.

Stably Pin1-overexpressing HTB-94 and human chondrocytes were established using MSCV-GFP retrovirus system (Lee et al., 2007). Briefly, Pin1 cDNA was subcloned into MSCV-GFP retroviral vector and phoenix cells (a packaging cell line) were transfected with MSCV-GFP (Control) or MSCV-Pin1-GFP (Pin1 overexpression) plasmid. Supernatants containing amphotrophic replication-incompetent retroviruses were collected and then stored at -80°C until required. 20% confluent HTB-94 cells and chondrocytes

obtained from osteoarthritic patients were multiply infected (12 times) with retrovirus particles. Intensities of infection were monitored by GFP-fluorescence and Western blot analysis using Pin1 antibody.

3. 9. Preparation of nuclear extract and Western blot analysis.

Cells were removed using a cell scraper and centrifuged at 2,500*g* for 5 min at 4°C. The cells were then swollen with 100μl of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1mM dithiothreitol and 0.5mM phenylmethylsulfonylfluoride]. Tubes were vortexed to disrupt cell membranes, and samples were incubated for 10 min on ice and then centrifuged for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 100μl of extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride], incubated for 30 min on ice, and centrifuged at 15,800*g* for 10 min; the supernatants containing the nuclear extracts were collected and stored at -80°C until required. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed as described previously (Lee et al., 2007). Cell lysates were fractionated by 10% gel

electrophoresis, and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently incubated with primary antibody, and then with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies. Finally, the membranes were developed using either 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium or using an ECL chemiluminescence detection kit.

3. 10. Gel shift assay.

Double-stranded DNA probes (2 *p*mole/ l) for the consensus sequences of AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') and NF-IL6 C/EBP binding site in *COX-2* gene (5'-CAGTCATTTCGTCACATGGG-3') were used for gel shift analyses after end-labeling the probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The reaction mixture contained 2 l of 5 × binding buffer with 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC, 50 mM Tris-Cl (pH 7.5), 10 μg of nuclear extracts, and sterile water to a total volume of 10 μl. Incubations were carried out at room temperature for 20 min by adding 1 μl probe (10^6 cpm) after a 10 min pre-incubation. The specificity of

DNA/protein binding was determined through competition reactions using a 10-fold molar excess of unlabeled oligonucleotides. Samples were loaded onto 5% polyacrylamide gels at 100V. After electrophoresis, the gels were removed, dried, and autoradiographed.

3. 11. Construction of a COX-2 promoter-luciferase construct and reporter gene assays:

To determine the transcriptional activity of the *COX-2* gene, we used the pGL-COX-2-574 luciferase reporter gene. To construct the luciferase (LUC) reporter gene plasmid, COX-2-LUC(-574), a DNA fragment containing -574 bp of 5'-flanking sequences and 113 bp of 5'-untranslated region (UTR) from the human *COX-2* gene was first amplified by PCR using a human genomic clone as the template. The PCR fragment was then cloned into pGL3-Basic (Promega, Madison, WI). Site-directed mutagenesis of NF-κB, CRE/AP-1, and NF-IL6/CEBP binding sites was performed using a LAPCR *in vitro* Mutagenesis Kit (TAKARA SHUZO Ltd., Japan) (Jeong et al., 2007). 1 μg of the plasmid was transfected into the cells using LipofectAMINE2000 (Invitrogen Corp., Carlsbad, CA) or Hilymax® reagent (Dojindo Molecular Technologies, Gaithersburg, MD) according to the manufacturer's instructions. After 6 h, the

transfection medium was replaced with the basal culture medium without serum and the cells were further incubated for 18 h. The luciferase activities in the cell lysates were then measured using a luminometer. The relative luciferase activity was calculated by normalizing the promoter-driven luciferase activity versus *hRenilla* luciferase or β-galactosidase.

3. 12. Construction of an iNOS Promoter-luciferase Construct and NF-κB reporter gene assays

To determine the transcriptional activity of *iNOS* gene, we used the pGL-miNOS-1588 luciferase reporter assay system. To generate the miNOS promoter-luciferase construct (pGL-miNOS-1588), mouse genomic DNA was isolated from mouse tail using the SV genomic DNA isolation kit (Promega, Madison, WI). The miNOS promoter region from –1588 bp to +165 bp was amplified by polymerase chain reaction (PCR) using specific primers (forward: 5'-GGTACCGACTTTGATATGCTGAAATCCATA-3'; reverse: 5'-AGATCTAGTTGACTAGGCTACTCCGTG-3') and ligated into pGEM-T easy vector (Promega, Madison, WI). The amplified product was subcloned into the KpnI/BglII site of pGL3-basic plasmid after confirming its DNA sequence by sequencing.

Cells were plated at a density of 3×10^5 cells/well in 12-well plate and transfected on the following day. A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were transiently transfected with 1μg of pGL-miNOS1588, pNF-κB-Luciferase, or pAP-1-Luciferase plasmid and 20 ng of the pRL-SV plasmid (Promega, Madison, WI) using the Genejuice® Reagent (Novagen, Madison, WI) and then exposed to LPS for 18 h. Firefly and *Renilla* luciferase activities in cell lysates were measured using a luminometer (Turner Designs; TD-20, CA). Relative luciferase activities were calculated by normalizing iNOS, NF-κB, or AP-1 promoter-driven firefly luciferase activities versus that of *Renilla* luciferase.

3. 13. Reverse transcription-polymerase chain reaction (RT-PCR).

The total RNA was isolated using total RNA isolation kit (RNAgents®, Promega, Madison, WI). The total RNA (1.0μg) obtained from the cells was reverse-transcribed using an oligo (dT) 18mer as a primer and M-MLV reverse transcriptase (Bioneer, Eumsung, Korea) to produce the cDNAs. PCR was performed using the selective primers for human TNF-α , IL-1β and S16 ribosomal protein (S16r) genes. The PCRs were carried out for 42

cycles using the following conditions: denaturation at 98°C for 10 sec, annealing at 50°C for 0.5 min, and elongation at 72°C for 1 min. The band intensities of the amplified DNAs were compared after visualization using FLA-7000 (Fuji film, Tokyo, Japan).

3. 14. Enzyme-linked imuunosorbent assay (ELISA).

Commercial ELISA kit (Cayman Chemical, Ann Arbor, MI) was used to determine prostaglandin E_2 (PGE2) concentrations in culture medium according to the manufacturer's protocols.

3.16. Statistics.

One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. The Newman-Keuls test was used for multi-group comparisons. Statistical significance was accepted for p values of <0.05.

Results

Part one

4. Novel Role of Pin1 in Rheumatoid Arthiritis

4.1. Pin1 induction in arthritic tissues and its role in proinflammatory protein expression.

To determine the Pin 1 is chronically expressed in arthritic lesions, we determined Pin1 levels by immunohistochemistry in the ankle tissues from CII-induced RA mice. Hind paw swelling and erythema was increased in all mice injected with CII (Fig1). While Pin1 antibody-positive staining was not detected in control tissues, Pin1 induction was highly found in the RA tissues, mainly distributed in chondrocytes, lymphocytes and fibroblasts (Fig. 2A, left). Interestingly, COX-2 staining results showed very similar patterns to Pin1 staining (Fig. 2A, right), which raised a possibility that COX-2 expression might be related with Pin1 existence in RA tissues. Western blot analysis was then performed using ankle tissue homogenates to confirm these results. As expected, COX-2 and Pin1 were concomitantly induced in RA tissue homogenates; whereas only a slight amount of COX-2 and Pin1 were detected in the control (Fig. 2B). To clarify the phenotypes of Pin1 overexpression in RA tissues,

we established stably Pin1 overexpressing HTB-94 cells, a human chondrocyte cell line (Pin1-HTB-94) using retroviral infections. In comparison to GFP-HTB-94 (GFP-overexpressing) cells, Pin1 expression was highly detected in Pin1-HTB-94 cells (Fig. 3A). Western blot analysis showed that COX-2 expression was up-regulated in Pin1-overexpressed HTB-94 cells (Fig. 3A). Since PGE2 is one of the stable autacoids produced by COX-2, we further examined PGE2 levels in culture medium. PGE2 production was 4.3 fold increased in Pin1-HTB-94 cells. Moreover, the protein or mRNA levels of iNOS, TNF-α and IL-1β, representative proinflammatory enzymes and cytokines, were also highly enhanced in Pin1-HTB-94 cells (Fig. 3B). We then established Pin1-overexpressing human primary chondrocytes using the ankle tissues obtained during surgery of osteoarthritic patient. Although the basal Pin1 and COX-2 expressions were seen in primary chondrocytes from osteoarthritic patient, and the stable Pin1 overexpression also potentiated COX-2 expression in the primary cultured human chondrocytes (Fig. 3C).

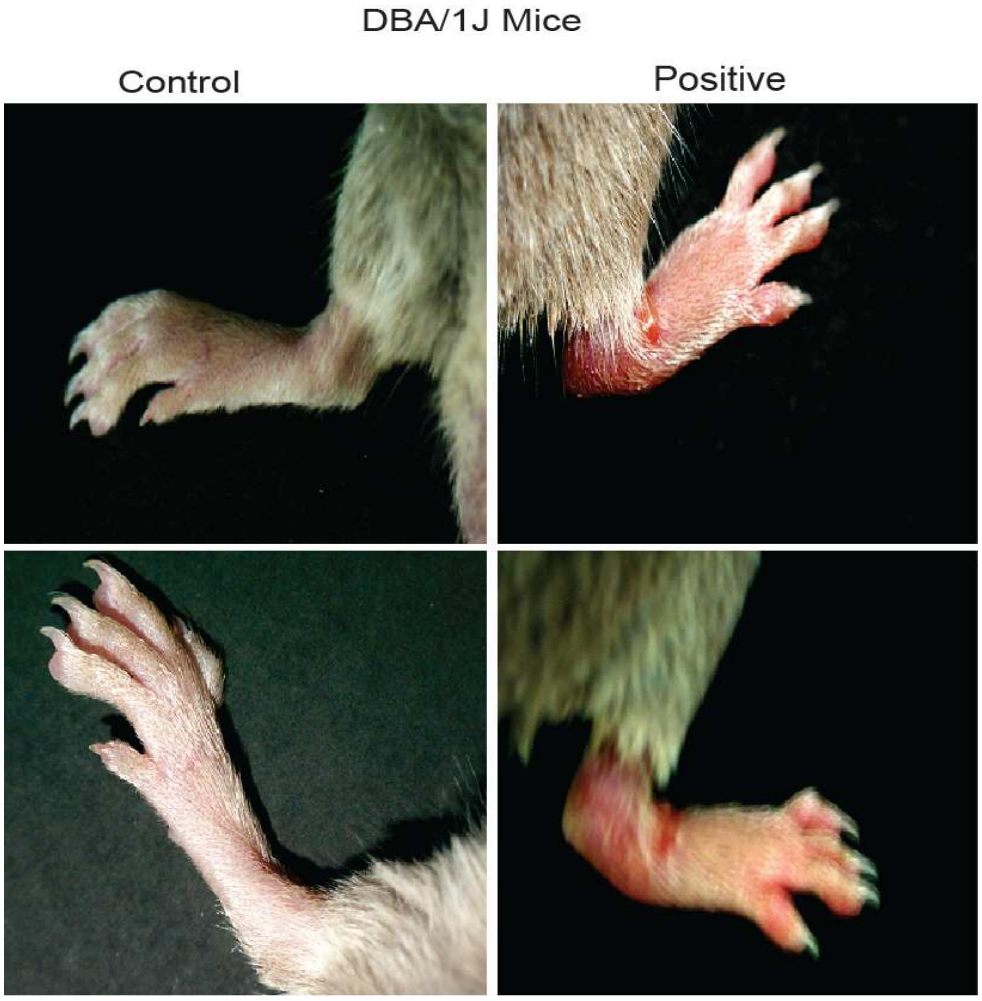


Fig. 1. Type II Collagen- induced Arthritis Model. 8 weeks old DBA/1J mice were used for the experiments. The mice were immunized intradermally as described in materials and method. On day 21, mice were boosted with second immunized intradermally with 100 μg CII dissolved in PBS and monitored arthritis development for 10 days.

Table 1. Effect of juglone on CII-induced RA

Group	Chronic Inflammation (Postive/ Total)	Ankylosis	Fibrosis	Articular cartilage loss	Synovialis proliferation	Bone damage
Control	0/4	0	0	0	0	0
CII	6/6	2.17±1.33	2.33±0.82	2.00±1.26	2.67±0.82	1.83±1.17
+ Juglone 1 mg/kg	4/4	0.75±1.50	1.25±0.96*	0.50±1.00*	1.00±0.82**	0.25±0.50*
+ Juglone 5 mg/kg	3/4	0.25±0.50*	1.50±1.00	1.00±0.82	1.25±1.26**	0.25±0.50*

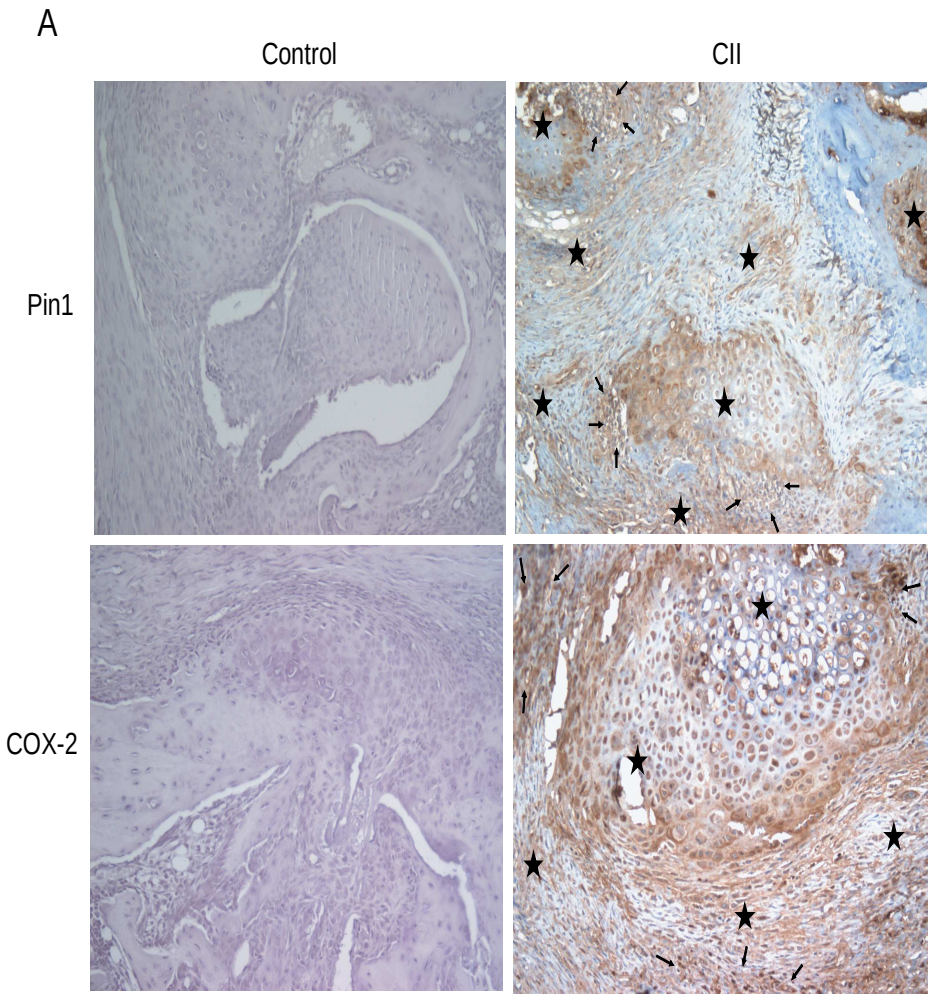
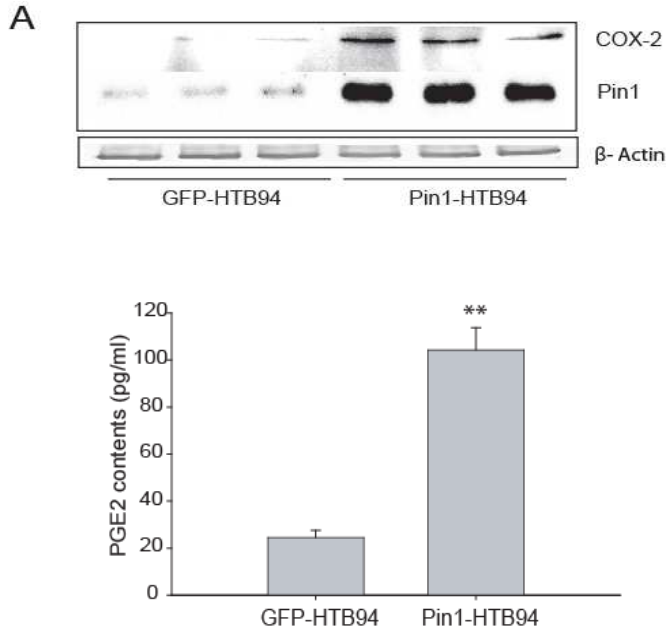
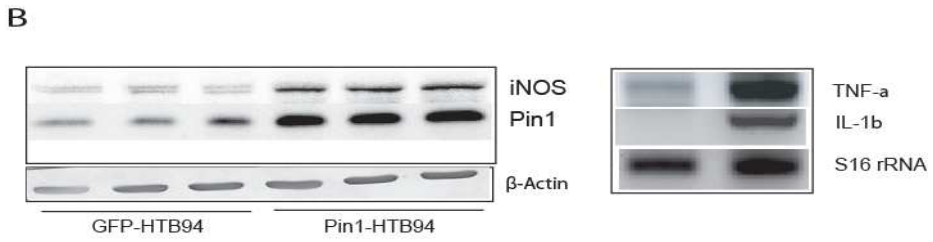

A
Control
CII
Pin1
COX-2
B
Pin1
COX-2
Control
CII

Fig. 2. Induction of Pin1 in ankle tissues of CII-injected DBA/1J mice. (A) Immunohistochemical staining of COX-2 and Pin1. Control mice ankle tissues showed no staining with COX-2 (upper right panel) but brown color staining with COX-2 in COll-II immunized mice (upper left panel). In the same way no staining with Pin1 in control mice ankle tissues (lower right panel) but well stain with Coll-II immunized mice (lower left panel). (B) Ankle tissues of both control and Coll-II mice were homogenized in cold PBS and COX-2 and Pin-1 immunoblot was performed. Coll-II immunized mice ankle tissues over express both COX-2 and Pin. All experiments were performed in duplicate.


A
COX-2
Pin1
β- Actin
GFP-HTB94
Pin1-HTB94
PGE2 contents (pg/ml)
120
100
80
60
40
20
0
**
GFP-HTB94
Pin1-HTB94


B
iNOS
Pin1
β-Actin
GFP-HTB94
Pin1-HTB94
TNF-a
IL-1b
S16 rRNA

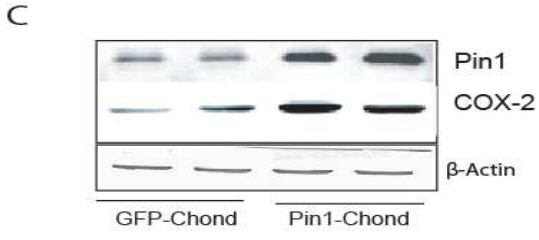

C
Pin1
COX-2
β-Actin
GFP-Chond
Pin1-Chond

Figure 3. Role of Pin1 overexpression in proinflammatory protein expression.

Stably Pin1-overexpressing HTB-94 and human chondrocytes were established using MSCV-GFP retrovirus system as described in materials and methods. HTB-94 cells and chondrocytes obtained from osteoarthritic patients were multiply infected (12 times) with retrovirus particles. Immunoblot analysis was performed with COX-2 antibody after the stable overexpression of GFP and Pin1 HTB94. PGE2 production in GFP and Pin1- HTB94 cells were determined after the 24 hours of serum deprived by using PGE2 ELISA ASSAY kit. (B) Immunoblot analysis of GFP and Pin1-HTB94 cells with iNOS and Pin1 antibodies (right panel) and mRNA of TNF-α, and IL1-β were determined by RT-PCR in GFP and Pin1-HTB94 cells, S16 ribosomal protein mRNA expression was comparable in samples. Immunoblot analysis of COX-2 and Pin1 expression in GFP and Pin1- Chondrocite cells after the stable overexpression of GFP and Pin1.

4.2. Pin1-dependent simultaneous activation of NF- B, CREB, C/EBP and AP-1 is required for the COX-2 expression.

Because COX-2-mediated PG production is considered as a representative inflammation index in RA, we chose *COX-2* gene expression as a model system for further experiments. Several studies have shown that CoX-2 expression is transcriptionally regulated by C/EBP, cAMO- response element binding protein (CREB), and NF-κB. To identify the role of each transcription factor in the regulation of COX-2 expression in Pin1-overexpressing chondrocytes, GFP- and Pin1-HTB-94 cells were transfected with the wild-type COX-2 promoter-luciferase chimeric construct that contained the 574-bp 5'-flanking region of human *COX-2* gene, or with C/EBP mutant with NF-IL6 site (-132/-124) mutation, NF-κB mutant with NF-κB site (-223/-214) mutation, or CRE/AP-1 mutant with CRE/AP-1 site (-59/-53) mutation(Tamura et al.,2003). When we determined promoter reporter activities, wild-type COX-2 promoter activity in Pin1-HTB-94 cells increased up to ~5-fold compared to GFP-HTB-94 cells. Each mutation of STAT-3, NF-κB or CRE/AP-1 significantly inhibited the Pin1-inducible reporter activity (Fig. 4A).Especially, the COX-2 promoter activity was most potently suppressed by NF-κB or

C/EBP binding site mutation (87% and 97% inhibition, respectively) (Fig. 4A). These results demonstrate that STAT-3, NF-κB and CRE/AP-1 elements are all essentially required for Pin1-mediated transactivation of the *COX-2* gene. Hence, we first compared NF-κB activity between GFP- and Pin1-HTB-94 cells. The NF-κB minimal reporter activity and nuclear p65 levels were higher in the Pin1-overexpressing cells (Fig. 4B). We also found that TPCK, a specific NF-κB inhibitor suppressed the Pin1-mediated COX-2 expression (Fig. 4C). It has been reported that Pin1 selectively increases nuclear p65 sequestration through the inhibition of p65 binding to I-κB (Ryo et al., 2003). Hence, it could be plausible that Pin1-mediated induction of proinflammatory cytokines partly result from p65/NF-κB activation.

We determined the activity of each transcription factor by using minimal reporter genes. The reporter activities of pCRE-Luc and pC/EBP-Luc were more than 13 fold and 4.2 fold enhanced in Pin1-HTB-94 cells versus control cells (Fig. 4A and 4B). Nuclear level of CREB was also sharply increased in Pin1-HTB-94 cells (Fig. 5A and 5B), but the increase intensities of C/EBPα and C/EBPβ were marginal (Fig. 5B).

We found that the AP-1 minimal reporter activity, AP-1 binding activity and nuclear distributions of c-Jun, c-Fos and Fra1 were significantly increased in the Pin1 overexpressed cells, though nuclear levels of JunB and JunD were not altered (Fig. 5C).

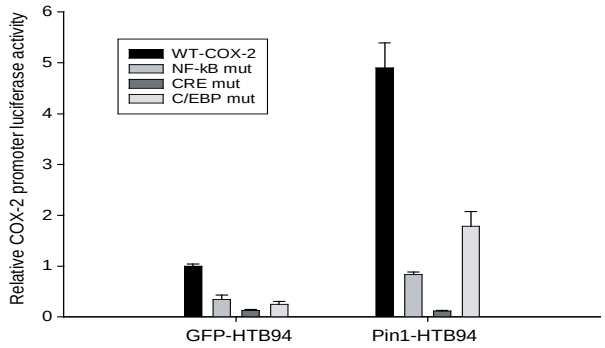
To study whether MAP kinase pathways are activated in Pin1-overexpressed cells, we measured the phosphorylated form of each MAP kinase. The level of active phosphorylated ERK was increased in Pin1-HTB94 cells compared to GFP-HTB94 cells (Fig. 5D). However, the phosphorylation intensities of JNK or p38 kinase were not affected by Pin1 overexpression (Fig. 5D). To further investigate whether blockade of the MAP kinase cascade led to a change in the expression of COX-2, we determined COX-2 expression changes in Pin1-HTB94 cells pretreated with MAP kinase inhibitors. Incubation of Pin1-HTB-94 cells with specific MAP kinase inhibitors (PD98059: ERK inhibitor; SP600125: JNK inhibitor, SB203580: p38 kinase inhibitor) for 36 h did not reduce the COX-2 protein levels (Fig. 5C). From these results, we can conclude that multiple transcription factors including NF-κB, C/EBP and CREB and AP-1 are complicatedly involved in the Pin1-dependent COX-2 expression in chondrocytes.

c-Jun and c-Fos-mediated AP-1 activity couples to Pin1

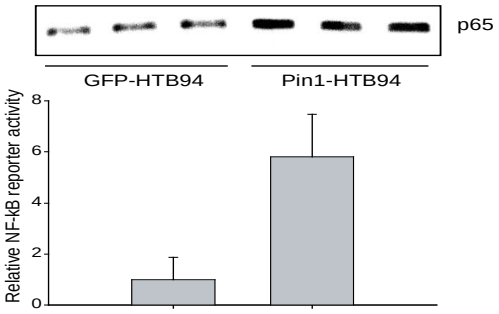
through its isomerase activity on phosphorylated c-Jun or c-Fos. In our experiments, nuclear levels of c-Jun, c-Fos and Fra1, but not those of JunB and JunD were increased in Pin1-HTB-94 cells, compared to GFP-HTB-94 cells. Since the enhanced AP-1 minimal reporter activity was almost completely suppressed by c-Jun siRNA, Pin1's target transcription factor for COX-2 gene transcription may be c-Jun. However, c-Jun siRNA treatment did not affect the COX-2 promoter activity in the Pin1-overexpressed HTB-94 cells, which imply that c-Jun/AP-1 activation in Pin1-HTB-94 cells is not essential for the COX-2 gene expression (fig. 5 E and F).

Several reports have shown that MAP kinase including ERK, JNK and p38 kinase regulates COX-2 expression. Here, we found that only ERK pathway was consistently activated in Pin1 everexpressed chondrocytes, but inhibition of JNK and p38 kinase as well as ERK did not cause the reduction of COX-2 expression. These data suggest that MAP kinases activities are not required for the Pin-1 mediated COX-2 expression.

A


Relative COX-2 promoter luciferase activity
6
5
4
3
2
1
0
WT-COX-2
NF-kB mut
CRE mut
C/EBP mut
GFP-HTB94
Pin1-HTB94

B


p65
GFP-HTB94
Pin1-HTB94
Relative NF-kB reporter activity
8
6
4
2
0

C

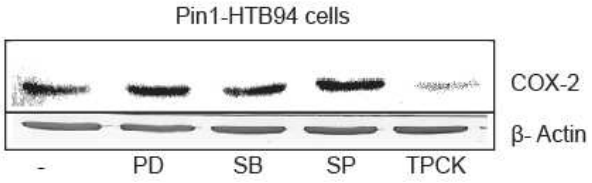
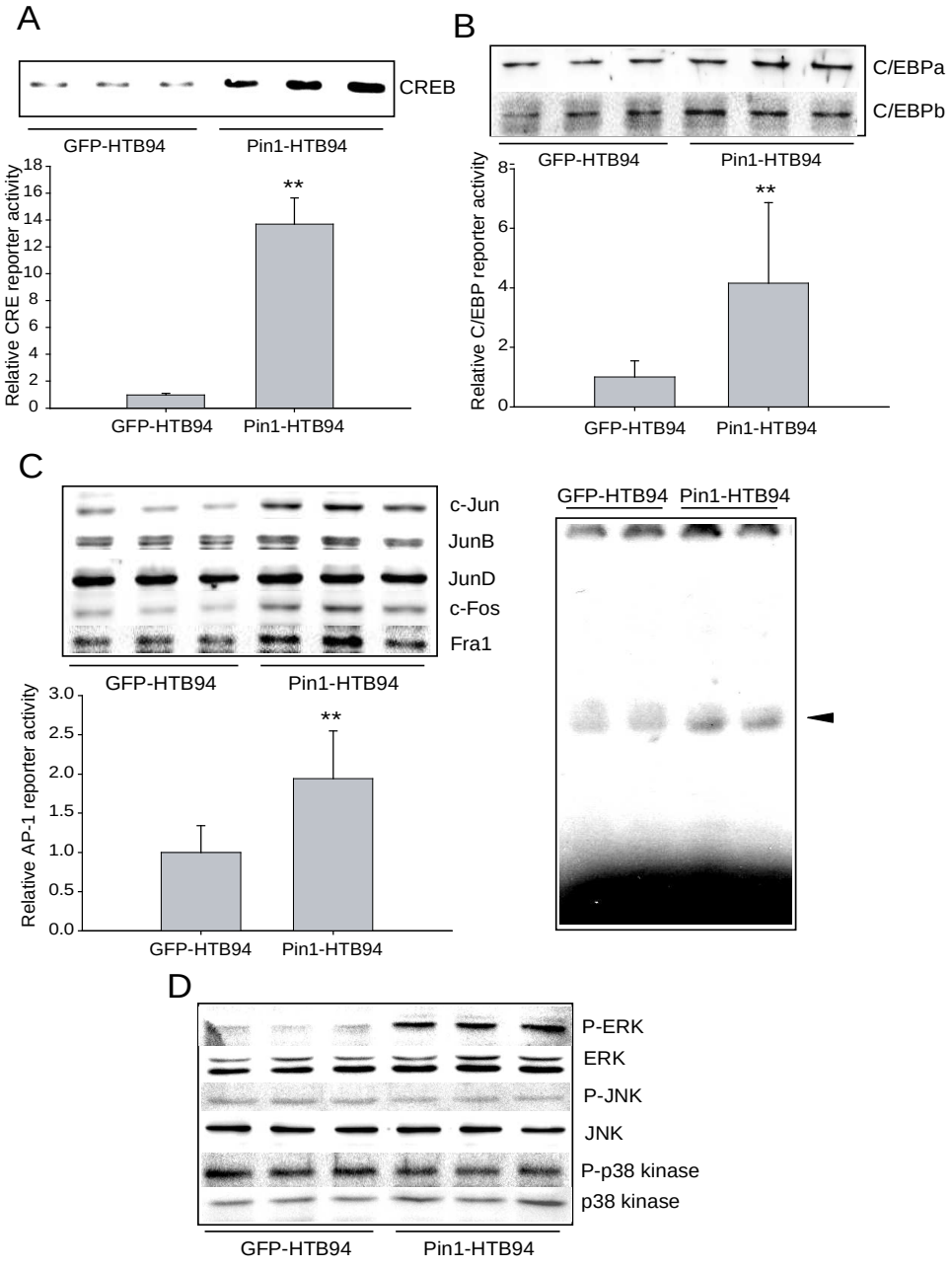
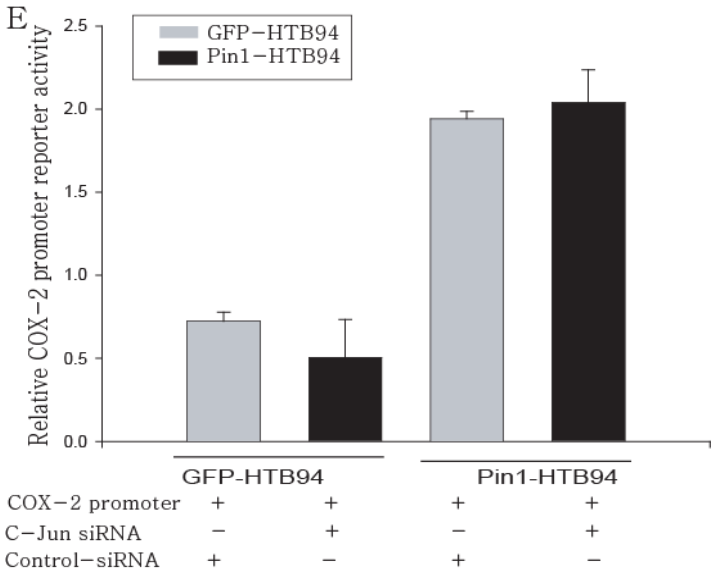

Pin1-HTB94 cells
COX-2
β- Actin
-
PD
SB
SP
TPCK

Figure 4. Pin1-dependent simultaneous activation of NF-κB, CREB, C/EBP and AP-1 is required for the COX-2 expression. (A) Essential role of NF- B, CREB/C/EBP, and Ap-1 activation in Pin1-inducible inflammatory gene expression. Induction of luciferase activity by Pin1- HTB94 cells as compared with GFP-HTB94 cells transiently transfected with pGL-COX-2-574, NF-κB mutant, C/EBP mutant or CRE mutant construct, was confirmed using a luminometer. Reporter gene activations were expressed as changes relative to b-galactosidase activity. (B) Nuclear translocation of P65 was determined in GFP and Pin1-HTB94 cells by immunochemically using specific antibody. (C) Immunoblotting of Pin1 HTB94 cells after the treatment of cells with different kinases inhibitors and NF-κB. The results shown represent the means ± SD of 3 separate experiments (significant as compared in GFP and Pin1 HTB94 cells reporter activity values of pGL-COX-2-574-transfected cells, $^{**}p < 0.01$).


A
CREB
GFP-HTB94
Pin1-HTB94
Relative CRE reporter activity
18
16
14
12
10
8
6
4
2
0
**
GFP-HTB94
Pin1-HTB94
B
C/EBPa
C/EBPb
GFP-HTB94
Pin1-HTB94
Relative C/EBP reporter activity
8
6
4
2
0
**
GFP-HTB94
Pin1-HTB94
C
c-Jun
JunB
JunD
c-Fos
Fra1
GFP-HTB94
Pin1-HTB94
Relative AP-1 reporter activity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
GFP-HTB94
Pin1-HTB94
GFP-HTB94
Pin1-HTB94
D
P-ERK
ERK
P-JNK
JNK
P-p38 kinase
p38 kinase
GFP-HTB94
Pin1-HTB94

E


GFP−HTB94
Pin1−HTB94
Relative COX−2 promoter reporter activity
2.5
2.0
1.5
1.0
0.5
0.0
GFP-HTB94
Pin1-HTB94
COX−2 promoter + + + +
C−Jun siRNA − + − +
Control−siRNA + − + −

F

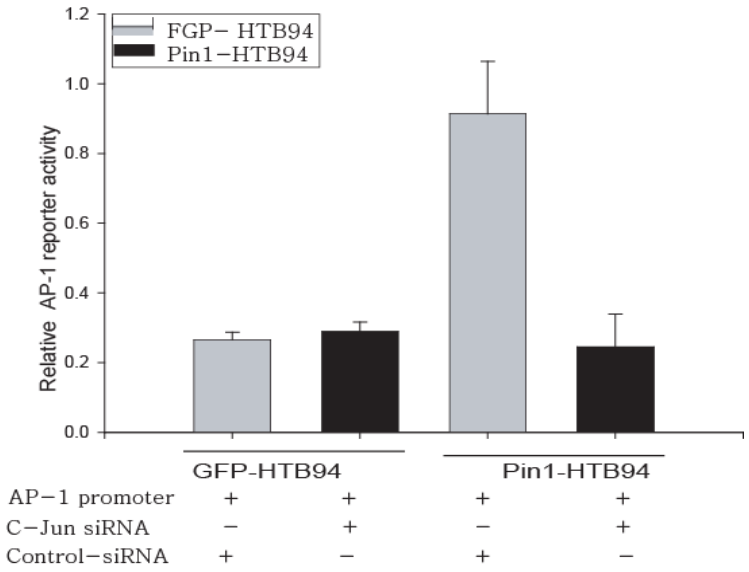

FGP− HTB94
Pin1−HTB94
Relative AP-1 reporter activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GFP-HTB94
Pin1-HTB94
AP−1 promoter + + + +
C−Jun siRNA − + − +
Control−siRNA + − + −

Figure.5. Pin1 Induction stimulates transcription of proinflammtory proteins. (A)Immunoblot analysis of GFP and Pin1-HTB94 cells after the 24 hour serum deprivation (upper panel) with CREB antibody (the nuclear levels of each transcription factor was determined immunochemically using specific antibodies), induction of luciferase activity by Pin1 overexpression in HTB94 cells transient transfected with CRE plasmid (lower panel). (B) Immunoblot analysis of GFP and Pin1 HTB-94 cells with C/EBP α and β antibodies (upper panel), induction of luciferase activity by Pin1 overexpression in HTB94 cells transient transfected with C/EBP plasmid (lower panel).(C) Immunoblot analysis of GFP and Pin1 HTB-94 cells with C-Jun, JunB, Jun D, c-Fos, and Fra1 antibodies (right upper panel) (the nuclear levels of each transcription factor was determined) immunochemically using specific antibodies, induction of luciferase activity by Pin1 overexpression in HTB94 cells transient transfected with Ap-1 minimal promoter plasmid (right lower panel) and Gel shift assay were performed with nuclear extracts prepared from GFP and Pin1- HTB 94 cells. All lanes were loaded with 10 μg of nuclear extracts and labeled with Ap-1 DNA consensus sequences (left panel). (D) Immunoblot analysis of MAP

kinases activity in GFP and Pin1-HTB94 cells with P- ERK, ERK, P-JNK, JNK, and P-P38 kinase and P38 kinase antibodies respectively in cell lysate. E) Effect of c-Jun siRNA over the COX-2 promoter activity. GFP and Pin1-HTB94 cells were transfected with COX-2 promoter plasmid and control and c-Jun siRNA (60pmole). F) Inhibition of AP-1 minimal promoter reporter activity after the transfection of cells with control and c-Jun siRNA (60 Pmole) respectively. The results shown represent the means ± SD of 3 separate experiments (significant as compared in GFP and Pin1 HTB94 cells reporter activity values of pGL-COX-2-574-transfected cells, $^{**}p < 0.01$).

4. 3. Juglone inhibits RA progress in CII-inducible DBA/1J mice and suppresses COX-2 expression in human primary cultured RA chondrocytes.

Next, we tested the effect of chemical Pin1 inhibitor, juglone on the CII-induced RA in DBA/1J mice. Intraperitoneal injection of juglone once another day started after booster injection of CII and continued for 10 days. We histopathologically evaluated RA grades of ankle joints by severity of inflammation, fibrosis, damages of articular cartilage and bone, and ankylosis after sacrificing the mice (Fig. 6A and Table 1). Juglone treatment (1 and 5 mg/kg) significantly inhibited the histological damage and cumulative arthritis injury scores, as compared with vehicle-treated CII-RA group (Table 1). Moreover, the enhanced COX-2 expression in the RA tissues was reversed in juglone-treated samples (Fig. 6B).

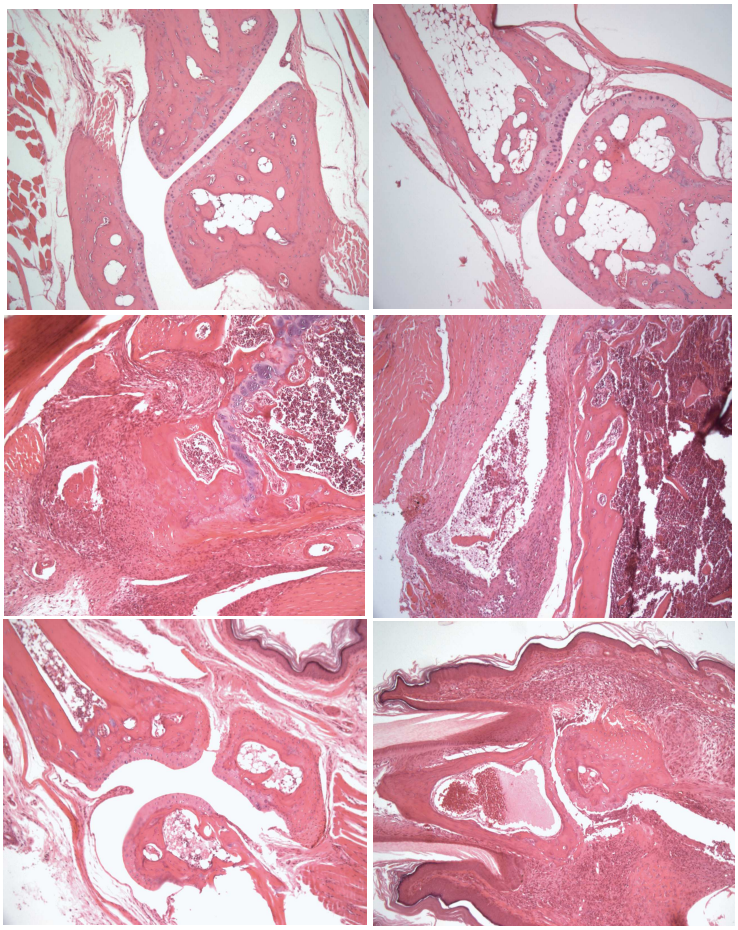
To finally prove whether Pin1 inhibition causes the down-regulation of proinflammatory mediators in human chondrocytes from RA patient, we determined the protein levels of COX-2 in the primary cultured chondrocytes from RA patient. The basal COX-2 expression was seen in chondrocytes obtained from RA patient and

pretreatment of the cells with juglone for 36 h blocked the basal expression of COX-2 in a concentration-dependent manner (Fig. 6C). These results imply that Pin1 could be a potential pharmacological target of RA progress in clinics.

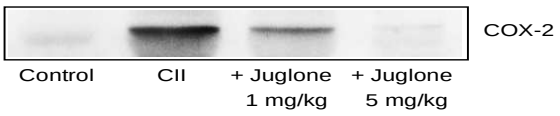
Taken together, Pin1 is up-regulated in the chondrocytes, lymphocytes and fibroblasts of RA lesions of CII-injected RA mice and the Pin1 overexpression results in the induction of proinflammatory proteins including COX-2, iNOS, TNF-α and IL-1β. Pin1-dependent COX-2 expression is associated with the simultaneous activations of NF-κB, C/EBP, CREB and AP-1. Pin1 may serve as a new therapeutic target of RA.

Although RA is one of the most frequent inflammatory diseases, the molecular pathogenesis of this disease has not been totally clarified. Data presented here indicate that Pin1 is induced in the lesion area of CII-mediated arthritis and plays a key role in the excess production of proinflammatory mediators including Prostaglandins, NO, TNF-α and IL-1β. Several mechanisms may be involved in the overproduction of these multiple proteins in response to Pin1 overexpression.

A


Control
CII
+ Juglone
1 mg/kg

B


COX-2
Control
CII
+ Juglone
1 mg/kg
+ Juglone
5 mg/kg

C

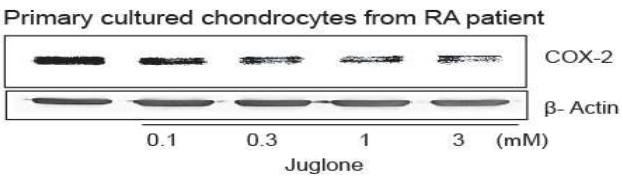

Primary cultured chondrocytes from RA patient
COX-2
β- Actin
0.1
0.3
1
3
(mM)
Juglone

Figure. 6. Pin1 inhibition suppresses COX-2 expression and arthritis progress. (A) Morphological changes of Collagen induced arthiritis, representative photographs by light microscopy with hematoxylin/eosin staining of ankle tissue sections from DBA/1J mice. The histological evaluation of ankle tissues, (n=6) treated with vehicle (upper panel), (N=6) II immunized with Coll-II (middle panel) revealed signs of severe arthritis, with inflammatory cell filtration and bone erosion and (n=6) injected interaperitonealy with 1mg/kg Juglone (3 times a week for 10 days) (lower panel) were significantly reduced in the inflammatory signs. These figures were representative of at least 3 experiments performed on different days. (B) Immunoblot of ankle tissues homogenate with cold PBS with COX-2 antibody after the treatment of DBA/1J mice with Juglone 1mg/kg for 10 days. (C) Immunoblot of primary cultured human chondrocyte from RA patients with COX-2 antibody after the 24 hour incubation with Juglone 0.1 to 3 μM/ml.

5. DISCUSSION

Collagen-induced arthritis is an experimental model of autoimmune disease, which can be induced in mice (Yoo et al., 1988), rats (Cuzzocrea et al., 1999a, b) and primates (Trentham, 1982) by immunization with type II native articular cartilage collagen (CII). The joint pathology associated with collagen induced arthritis is similar to the one observed in patients with RA (Stuart et al., 1982a, b). Both cellular and humoral immune responses to CII are involved in the pathogenesis of collagen induced arthritis. Mice injected with type II collagen (CII) induce polyarthritis (Svensson et al., 1998). Here we established the DBA/1J mice as a collagen induced arthritis model for the further experiments.

Pin1 is believed as one of pathological mediator in neurodegenerative disease (Balastik et al., 2007). Pin1 inhibition significantly inhibits eosinophilic inflammation in vitro and in vivo. Pin1 knockout mice showed the reduced expression of TGF- β1 after the allergen –sensitization (Zhong-Jian et al., 2008). Here we first time document a hypothesis that Pin1 is chronically expressed in arthritic lesions, we determined Pin1 levels by immunohistochemistry in the ankle tissues from CII-induced RA mice. Pin1 induction was

highly found in the RA tissues, mainly distributed in chondrocytes, lymphocytes and fibroblasts. It has been shown that COX-2 is highly expressed in human and animal arthritic tissues (Martel-Pelletier et al., 2003). In animal models of arthritis, COX-2 is highly expressed and is thought to be responsible for the increase in PG production in these animals (Anderson et al., 1996). In humans, COX-2 overexpression has been demonstrated in osteoarthritis (OA)–affected cartilage (Amin et al., 1997) and in synovial tissue from patients with RA (Kang et al., 1996). From these evidences it is proved that COX-2 in the most important target of inflammatory diseases like as arthritis. We also demonstrated that, COX-2 staining showed the similar pattern like Pin1 which raised a possibility that COX-2 expression might be related with Pin1 existence in RA tissues. In our western blotting result of ankle tissue homogenates showed the COX-2 and Pin1 concomitantly induced in RA tissues. For the further clarification of this result, we established the stably overexpressed Pin 1 in RA tissues and HTB-94, a human chondrocyte human primary cell line (Pin1- HTB94 using retroviral infections. We found that as compaired with GFP-HTB-94 (GFP-overexpressing) cells, Pin1 expression was highly detected in Pin1-HTB-94 cells. COX-2

expression was up-regulated in the similar pattern in Pin1- HTB94 cells. PGs influence the immune response mediated through mature B and T lymphocytes. PGE2 shifts the balance of the cellular response from Th1 to Th2 by inhibiting interleukin-2 and enhancing IL-4 production (Betz et al., 1991; Van der Pouw et al., 1995). Since PGE2 is one of the stable autacoids produced by COX-2, we further examined PGE2 levels in culture medium. PGE2 production was 4.3 fold increased in Pin1-HTB-94 cells. We then established Pin1-overexpressing human primary chondrocytes using the ankle tissues obtained during surgery of osteoarthritic patient. Although the basal Pin1 and COX-2 expressions were seen in primary chondrocytes from osteoarthritic patient, and the stable Pin1 overexpression also potentiated COX-2 expression in the primary cultured human chondrocytes.

Pro-inflammatory cytokines TNF-α and IL-1β involve in the extension of local and systemic inflammatory process (Deleuran et al., 1992; Westacott *et al*., 1990; Feldmann *et al*., 1990; Shinmei et al., 1989). Here we confirmed that the protein or mRNA levels of iNOS, TNF-α and IL-1β, representative proinflammatory enzymes and cytokines were also highly enhanced in Pin1-HTB-94 cells.

Production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage (Cuzzocrea et al., 1998a; 1999a; Oyanagui, 1994; Salvemini et al., 1998). NO play an important role in the pathophysiology of inflammation (Brahn *et al*., 1998b; Ialenti et al., 1993). We demonstrate here that the protein or mRNA levels of iNOS, TNF-α and IL-1β , representative proinflammatory enzymes and cytokines, were also highly enhanced in Pin1-HTB-94 cells.

Several studies have shown that COX-2 expression is transcriptionally regulated by C/EBP, cAMP-response element binding protein (CREB), and NF-κB and that these transcription factors are synergistically or independently involved in *COX-2* gene expression (Thomas et al.,2000, Tamura et al.,2003 Wardlaw et al., 2002). C/EBP transcription factors are involved in the regulation of gene transcription by IL-6 and they control inflammation (Poli et al., 1998). The C/EBP family includes three main members: C/EBP-α, C/EBP-β, and C/EPB-δ. IL-1β and C/EBP are positively regulated in the interaction with NF-κB (Lee et al., 1998; Jones et al., 1997). However, induction of C/EBP binding to DNA by proinflammatory cytokines correlates with the accumulation of prostaglandin E2, and both effects are reversed by

anti-inflammatory cytokines (Alaaeddine et al.,1999]. C/EBP factors act with NF-κB to induce the transcription of many acute-phase response genes in response to proinflammatory cytokines, and this effect is based on direct protein–protein interactions (Stein et al., 1993; Diehl et al., 1994; Kravchenko et al., 2003). We determined the activity of each transcription factor by using minimal reporter genes. The reporter activities of pCRE-Luc and pC/EBP-Luc were x.y and 4.2 fold enhanced in Pin1-HTB-94 cells versus control cells. Nuclear level of CREB was also sharply increased in Pin1-HTB-94 cells, but the increase intensities of C/EBP and C/EBP were marginal. To identify the role of each transcription factor in the regulation of COX-2 expression in Pin1-overexpressing chondrocytes, GFP- and Pin1-HTB-94 cells were transfected with the wild-type COX-2 promoter-luciferase chimeric construct that contained the 574-bp 5'-flanking region of human *COX-2* gene, or with C/EBP mutant with NF-IL6 site (-132/-124) mutation, NF-κB mutant with NF-κB site (-223/-214) mutation, or CRE/AP-1 mutant with CRE/AP-1 site (-59/-53) mutation (Tamura et al.,2003). When we determined promoter reporter activities, wild-type COX-2 promoter activity in Pin1-HTB-94 cells increased up to ~5-fold compared to GFP-HTB-94 cells. Each mutation of STAT-3,

NF-κB or CRE/AP-1 significantly inhibited the Pin1-inducible reporter activity. Especially, the COX-2 promoter activity was most potently suppressed by NF-κB or C/EBP binding site mutation (87% and 97% inhibition, respectively). These results demonstrate that STAT-3, NF-κB and CRE/AP-1 elements are all essentially required for Pin1-mediated transactivation of the *COX-2* gene. Hence, we first compared NF-κB activity between GFP- and Pin1-HTB-94 cells. The NF-κB minimal reporter activity and nuclear p65 levels were higher in the Pin1-overexpressing cells. We also found that TPCK, a specific NF-κB inhibitor suppressed the Pin1-mediated COX-2 expression. It has been reported that Pin1 selectively increases nuclear p65 sequestration through the inhibition of p65 binding to I-κB (Ryo et al., 2003). Hence, it could be plausible that Pin1-mediated induction of proinflammatory cytokines partly result from p65/NF-κB activation.

In the promoter region of the COX-2 gene, two NF-κB consensus sequences are located and the expressions of Pin1-inducible proinflammatory genes including iNOS, IL-1β and TNF-α gene are mainly dependent on NF-κB activation (Neuton et al., 1997; Tak and Firestein, 2001; Xiao and Ghosh., 2005).Since C/EBP and CRE/AP-1 elements are also involved in cytokine- or ultraviolet B-

inducible COX-*2* gene transcription (Tang et al., 2001; Thomas et al., 2000). We determined COX-2 and PG production is considered as a representative inflammation index in RA, we select COX-2 gene expression as a model system for the further experiments.Several studies have shown that CoX-2 expression is transcriptionally regulated by C/EBP, cAMP- response element binding protein (CREB), and NF- κB.

We confirmed that the AP-1 minimal reporter activity, AP-1 binding activity and nuclear distributions of c-Jun, c-Fos and Fra1 were significantly increased in the Pin1 overexpressed cells, though nuclear levels of JunB and JunD were not altered. Either c-Jun or c-Fos are known to be *cis/trans* isomerized by Pin1 and it has been suggested that Pin1 plays a key role in AP-1-dependent gene transcription upon phosphorylation by the MAP kinase family (Wulf et al., 2001; Monje et al., 2005). c-Jun activation by platelet-derived growth factor or serum induced COX-2 via CRE/AP-1 binding site (Xie and Herschman, 1996). Several reports have shown that MAP kinases including ERK, JNK and p38 kinase regulates COX-2 expression through the regulation of NF-κB, C/EBP or CREB in diverse pathological conditions (Lee et al., 2007; Ki et al., 2007; Han

et al., 2004).Both the phosphorylation and the expression of c-Fos and c-Jun are controlled by MAP kinase family (Chun and Surh, 2004). It has been also shown that MAP kinases play a crucial role in regulating COX-2 expression (Chen et al., 2001). We found that the level of active phosphorylated ERK was increased in Pin1-HTB94 cells compared to GFP-HTB94 cells. However, the phosphorylation intensities of JNK or p38 kinase were not affected by Pin1 overexpression. For the further confirmation whether blockade of the MAP kinase cascade led to a change in the expression of COX-2, we determined COX-2 expression changes in Pin1-HTB94 cells pretreated with MAP kinase inhibitors. Incubation of Pin1-HTB-94 cells with specific MAP kinase inhibitors (PD98059: ERK inhibitor; SP600125: JNK inhibitor, SB203580: p38 kinase inhibitor) for 36 h did not reduce the COX-2 protein levels. From these results, we can conclude that multiple transcription factors including NF-κB, C/EBP and CREB and AP-1 are complicatedly involved in the Pin1-dependent COX-2 expression in chondrocytes. Here, we found only ERK pathway was consistently activated in Pin1-overexpressed chondrocytes, but inhibition of JNK and p38 kinase as well as ERK did not cause the reduction of COX-2 expression. These data suggest

that MAP kinases activities are not required for the Pin1-mediated COX-2 expression.

In our experiments, nuclear levels of c-Jun, c-Fos and Fra1, but not those of JunB and JunD were increased in Pin1-HTB-94 cells, compared to GFP-HTB-94 cells. Since the enhanced AP-1 minimal reporter activity was almost completely suppressed by c-Jun siRNA, Pin1's target transcription factor for *COX-2* gene transcription may be c-Jun. However, c-Jun siRNA treatment did not affect the COX-2 promoter activity in the Pin1-overexpressed HTB-94 cells, which imply that c-Jun/AP-1 activation in Pin1-HTB-94 cells is not essential for the *COX-2* gene expression.

NF-κB which forms a homo or heterodimer complex plays an important role in the regulation of various genes responsible for the stimulation of inflammation reactions. Several studies have shown that COX-2 and iNOS expression is transcriptionally regulated by C/EBP, CREB as well as NF-κB and that these transcription factors may be synergistically or independently involved in the expression of these gene expression (Draska., 1999; Kinugawa et al., 1997; Wardlaw et al., 2002) and many phytochemicals inhibiting NF-κB has been proven to effective against RA in animal and human studies (Bremner et al.,

2002; Kauss et al., 2008). It has been shown that Pin1 bind to the phosphorylated Thr254-Pro of p65 and subsequently inhibits Inhibitor-κB binding, which finally result in the increased nuclear localization and NF-κB activity (Ryo et al., 2003). We also revealed that either *COX-2* gene expression or its promoter activity was dependent on NF-κB activity. Thus, NF-κB activation in Pin1-activated chondrocytes is obviously associated with the overwhelmed production of proinflammatory mediators during RA progress.

The present data by COX-2 promoter mutation, minimal reporter genes and Western blot analyses revealed that CRE and C/EBPs are consistently activated by Pin1 and also demonstrate that C/EBPs and CREB are transcriptionally active to increase COX-2 expression in Pin1-overexpressed chondrocytes. In silico analysis revelaed that C/EBP variants contain Ser/Thr-Pro-rich segmants and speculated that consensus phosphorylation sequences for Pin1 or Polo-like kinase was located in a highly conserved region of transactivation domains of C/EBP (Miller, 2006). Although there is still no report showing that transcriptional activity of CREB is dependent on Pin1, it could be plausible that the transcriptional activities of C/EBP and CREB are controlled by Pin1.

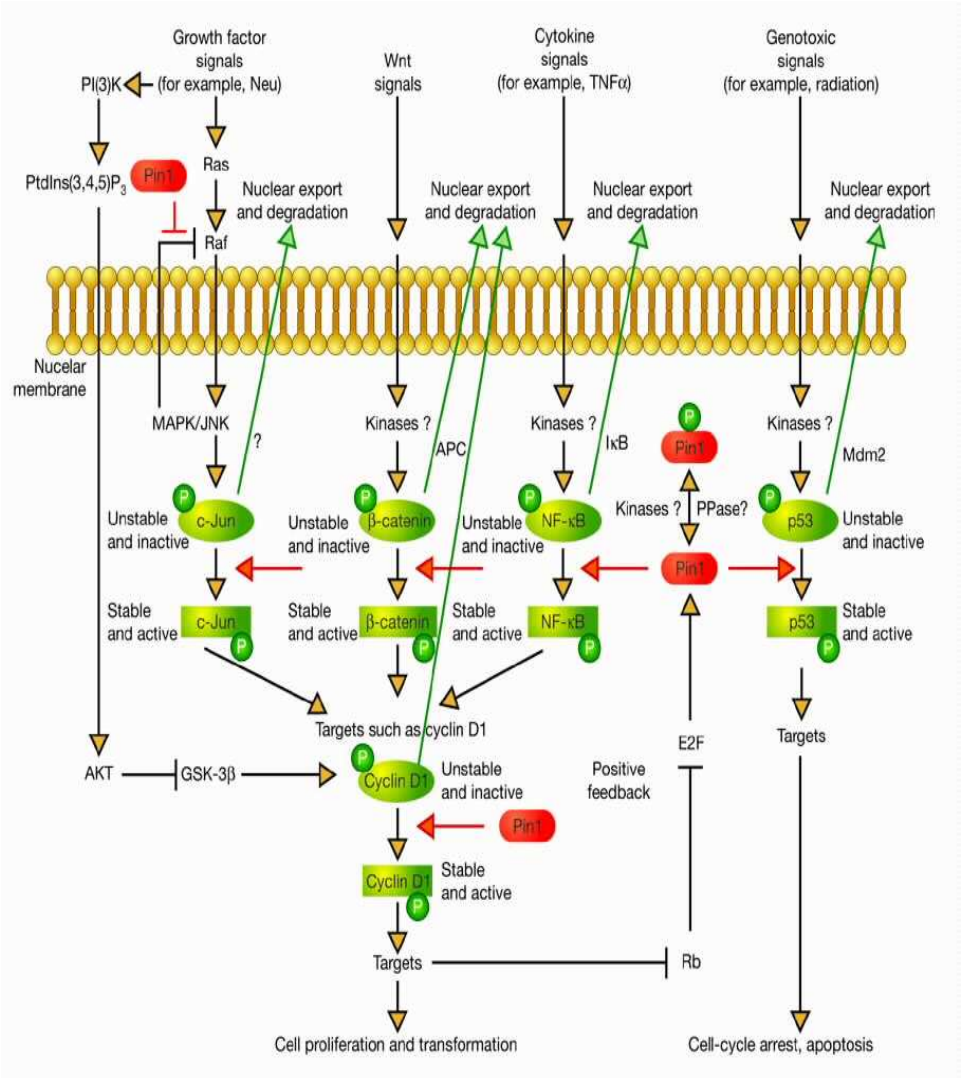
The juglone covalently inactivates a unique cysteine residue in the active site of Pin1 isomerase (Hennig et al., 1998). Juglone suppress the Pin1 and eosinophilic pulmonary inflammation, TGF-β1 and collagen expression, and airway remodeling (Shen et al., 2008).Pin1 induce the type 1 immune response and suppression of Pin1 by pharmacologic or genetic means greatly attenuated IFN-γ, IL-2 and CXCL-10 mRNA stability, accumulation and protein expression after cell activation (Esnault et al., 2007). We found that intraperitoneal injected juglone, significantly inhibited the histological damage and cumulative arthritis injury scores, as compared with vehicle-treated CII-RA group (Table 1). Moreover, the enhanced COX-2 expression in the RA tissues was reversed in juglone-treated samples.

To finally prove whether Pin1 inhibition causes the down-regulation of proinflammatory mediators in human chondrocytes from RA patient, we determined the protein levels of COX-2 protein levels in the primary cultured chondrocytes from RA patient. The basal COX-2 expression was seen in chondrocytes obtained from RA patient and pretreatment of the cells with juglone for 36 h blocked the basal expression of COX-2 in a concentration-dependent manner. These results imply that Pin1 could be a potential pharmacological

target of RA progress in clinics.

Taken together, Pin1 is up-regulated in the chondrocytes, lymphocytes and fibroblasts of RA lesions of CII-injected RA mice and the Pin1 overexpression results in the induction of proinflammatory proteins including COX-2, iNOS, TNF-α and IL-1β. Pin1-dependent COX-2 expression is associated with the simultaneous activations of NF-κB, C/EBP, CREB and AP-1. Pin1 may serve as a new therapeutic target of RA. Although RA is one of the most frequent inflammatory diseases, the molecular pathogenesis of this disease has not been totally clarified. Data presented here indicate that Pin1 is induced in the lesion area of CII-mediated arthritis and plays a key role in the excess production of proinflammatory mediators including prostaglandins, NO, TNF-α and IL-1β. Several mechanisms may be involved in the overproduction of these multiple proteins in response to Pin1 overexpression. In this study, stable Pin1 overexpression caused both the sustained nuclear translocation of p65 and the increase in NF-κB-driven transcription.

6. Signal transduction in Pin1 pathway



Wulf et al., Nature Cell Biol.

Part 2

7. Anti inflammatory effects of some phytochemicals

7. 1. Effects *Taiwaniaflavone,* 4-hydroxykobusin and 2′, 8”-biapigenin on the induction of iNOS by LPS

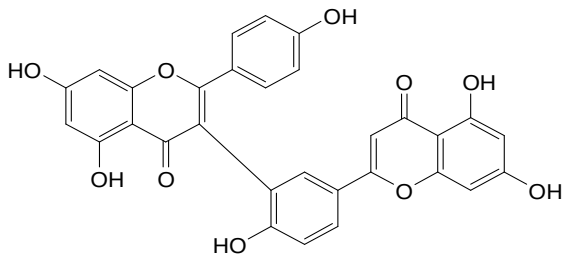
The chemical structure of taiwaniaflavone, 4-hydroxykobusin and 2’, 8”-biapigenin is presented in Fig 7A, B, and And C. Initially we measured the cytotoxicity of taiwaniaflavone and 4-hydroxykobusin to RAW264.7 cells by MTT assay. Cell viability was not significantly altered by taiwaniaflavone and 4-hydroxykobusin at up to 100 mM (Fig. 8, A&B). Thus, we treated cells with taiwaniaflavone and 4-hydroxykobusin in the concentration range 3-100 mM during subsequent experiments. To assess the NO-blocking effect of taiwaniaflavone, 4-hydroxykobusin and 2’, 8”-biapigenin, we monitored nitrite levels in culture media after stimulating cells with LPS (1 μg/ml) in the presence or absence of taiwaniaflavone, 4-hydroxykobusin and 2’, 8”-biapigenin for 48 h. LPS stimulation caused a significant accumulation of nitrite in culture media at 12 h (4.3 fold), 24 h (12.5 fold) and 48 h (19.9 fold) (Fig.9A) by twainiaflavone. However, pretreatment with taiwaniaflavone (10-100 μM) significantly attenuated LPS-induced

nitrite production in a concentration-dependent manner (Fig. 9A). LPS stimulation caused a significant increase of nitrite in culture media at 12 h (2.5 fold), 24 h (6.2 fold) and 48 h (7.3 fold) (Fig. 9A). This enhancement in NO production was significantly suppressed by 4-hydroxykobusin in a concentration dependent manner. Especially, LPS-inducible NO production was ~90% blocked by 100 M 4-hydroxykobusin (Fig. 9B). To determine the NO-blocking effect of 2',8'-biapigenin, we monitored nitrite levels in culture media after stimulating cells with LPS in the presence or absence of 2',8'-biapigenin for 48 h. LPS (1 μg/ml) significantly increased NO production from 12 h (4.3 fold) to 48 h (19.9 fold) (Fig. 9B). 2', 8"-biapigenin significantly inhibited LPS-induced NO production in a concentration-dependent manner (10-100 μM) (Fig. 9C).

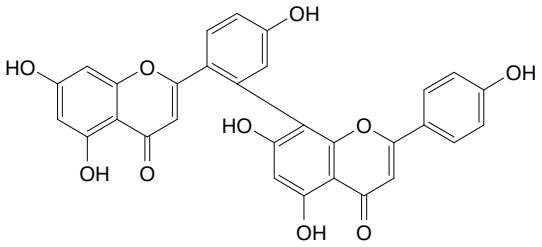
We then examined whether the inhibition of NO production by taiwaniaflavone is due to *iNOS* transcription. Western blot analysis using iNOS-specific antibody showed that exposure of RAW264.7 cells to LPS (1 μg/ml) for 12 h increased iNOS protein levels versus un-stimulated controls. Moreover, taiwaniaflavone (10-100 μM) significantly reduced iNOS protein expression, and in particular, 30 or 100 μM of taiwaniaflavone completely inhibited

iNOS protein upregulation (Fig. 10A). These results show that de novo iNOS synthesis was suppressed by taiwaniaflavone in LPS-activated macrophages. We then investigated whether the inhibition of NO formation by 4-hydroxykobusin was the result of the inhibition of iNOS gene expression. The inhibitory effects of different concentrations of 4-hydroxykobusin on iNOS protein expression induced by LPS (1 μg/ml) were estimated. Western blot analysis using iNOS-specific antibody showed that exposure of RAW264.7 cells to LPS (1 μg/ml) for 18 h increased iNOS protein levels versus un-stimulated controls (Fig. 10B). Pretreatment of RAW264.7 cells with 4-hydroxykobusin (10 min) significantly inhibited iNOS protein expression at 30-100 μM (Fig. 10B). These results suggest that 4-hydroxykobusin is effective to block iNOS induction and NO production in macrophages. To examine whether the blocking of NO production by 2',8'-biapigenin was mediated by a process involving iNOS gene expression, iNOS protein and mRNA levels were measured by Western blotting and RT-PCR analyses, respectively. LPS (1 μg/ml) treatment increased the level of iNOS protein and 30 or 100 μM 2', 8"-biapigenin almost completely inhibited this increase in iNOS protein level (Fig. 10C). Glyceraldehyde 3-phosphate

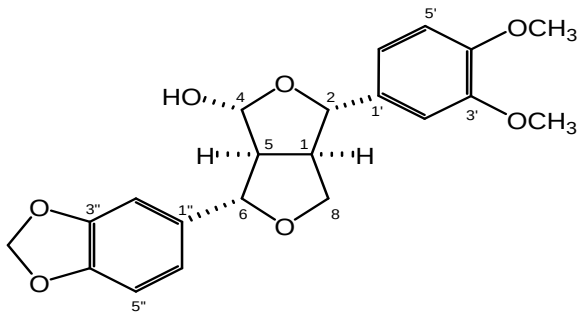
dehydrogenase (GAPDH) levels were comparable among the samples (Fig. 10C).Consistent with the Western blot result, LPS-inducible iNOS mRNA levels were also suppressed by 30 μM 2', 8"-biapigenin in macrophages (Fig. 10D). The mRNA level of S16 ribosomal protein was used as an internal loading control (Fig. 10D). To test whether 2', 8"-biapigenin inhibit iNOS expression in other macrophage cell line, we used J774.A1 cells (a murine macrophage cell line). The levels of iNOS was increased in J774.A1 cells incubated with tumor necrosis factor-α (20 ng/ml) and interferon-γ (20 ng/ml) and this increase was also completely reversed by 10 μM 2', 8"-biapigenin (Fig.10D). These results suggest that iNOS gene transcription is suppressed by 2', 8"-biapigenin in activated macrophages



A Taiwaniaflavone



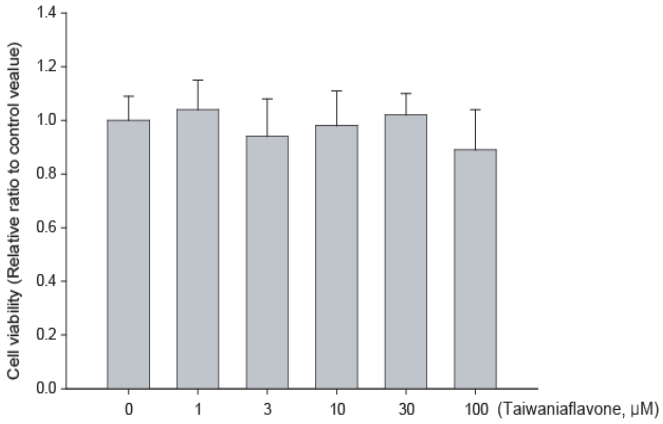
B 2',8''-biapigenin



C **4-Hydroxykobusin**

Fig. 7. Chemical structure of (A) Taiwaniaflavone, 2′, 8″ Biapegenin, and 4- Hydroxykobusin. Structure of 4-Hydroxykobusin Isolated from *Geranium thunbergii (*Geraniaceae)

A)


Cell viability (Relative ratio to control veaiue)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
3
10
30
100
(Taiwaniaflavone, μM)

B)

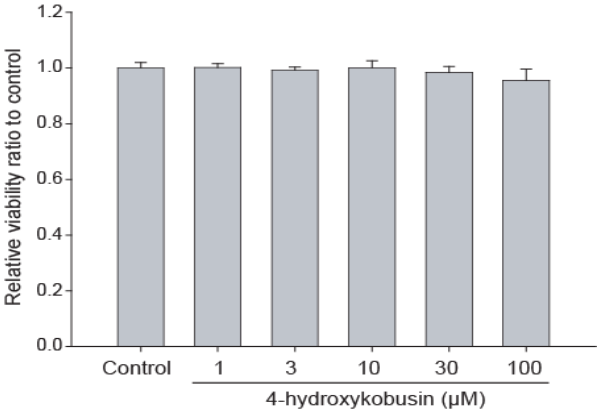
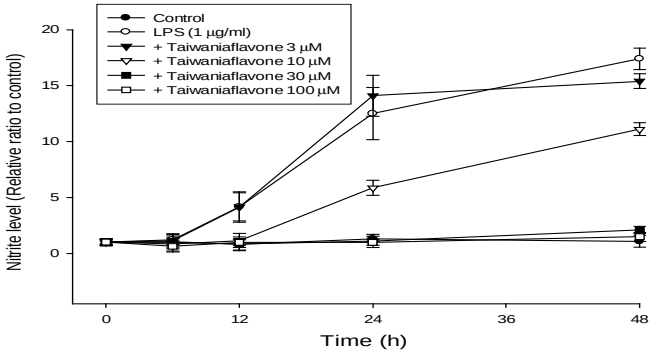
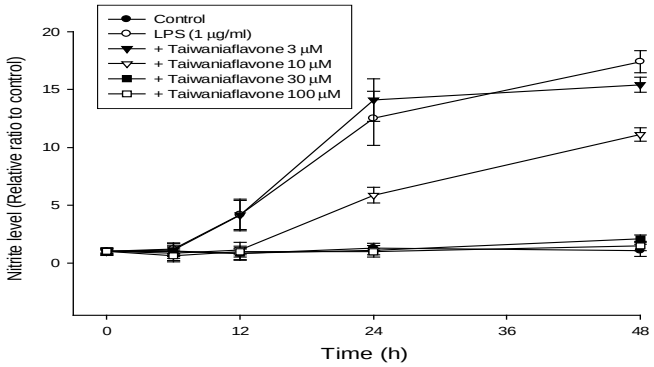

Relative viability ratio to control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
1
3
10
30
100
4-hydroxykobusin (μM)

Fig. 8. (A)Effect of taiwaniaflavone on cell viability. RAW264.7 cells were incubated in the presence or absence of 1–100 μM taiwaniaflavone. Cell viabilities were determined by MTT assay. Data represent the means §SD of eight different samples. (B) Effect of 4-Hydroxykobusin on Cell Viability RAW264.7 cells were incubated in the presence or absence of 1-100μM 4-hydroxykobusinfor 24 h. Cell viabilities were determined by MTT assay. Data represent the means_§SD of 8 different samples

A)


Control
LPS (1 μg/ml)
+ Taiwaniaflavone 3 μM
+ Taiwaniaflavone 10 μM
+ Taiwaniaflavone 30 μM
+ Taiwaniaflavone 100 μM
Nitrite level (Relative ratio to control)
20
15
10
5
0
0
12
24
36
48
Time (h)

B)


Control
LPS (1 μg/ml)
+ Taiwaniaflavone 3 μM
+ Taiwaniaflavone 10 μM
+ Taiwaniaflavone 30 μM
+ Taiwaniaflavone 100 μM
Nitrite level (Relative ratio to control)
20
15
10
5
0
0
12
24
36
48
Time (h)

C)

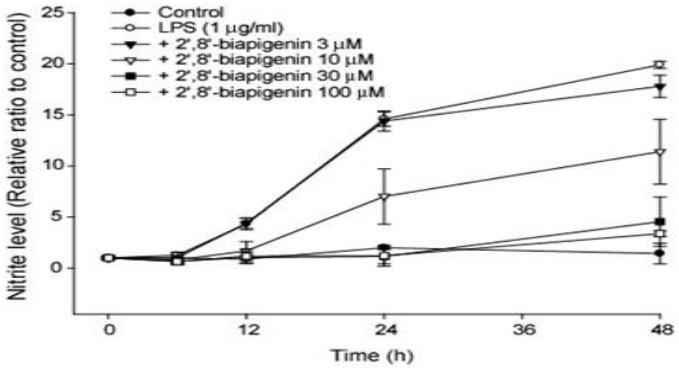
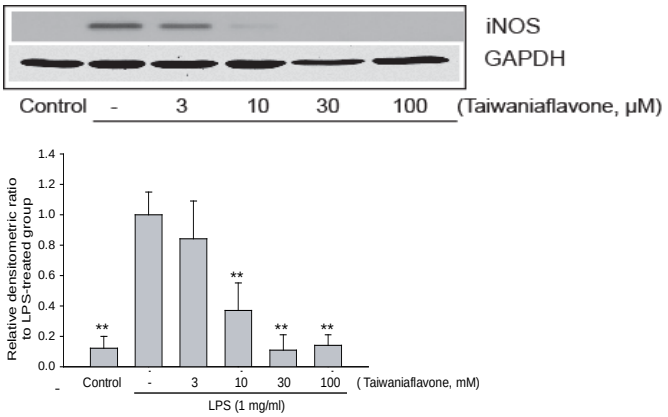
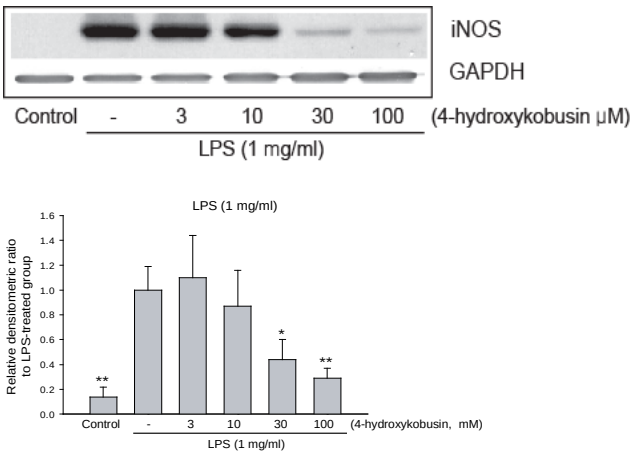

Control
LPS (1 μg/ml)
+ 2',8'-biapigenin 3 μM
+ 2',8'-biapigenin 10 μM
+ 2',8'-biapigenin 30 μM
+ 2',8'-biapigenin 100 μM
Nitrite level (Relative ratio to control)
25
20
15
10
5
0
0
12
24
36
48
Time (h)

Fig. 9. Effects of Natural Compunds on LPS-Induced NO Production. (A) The effects of taiwaniaflavone on LPS-induced NO production. RAW264.7 cells were incubated in a medium containing taiwaniaXavone (3, 10, 30, and 100 μM) for 10 min and then treated with LPS (1 μg/ml). The amount of nitrite in medium was monitored for 48 h. (B) Effect of 4-hydroxykobusin on LPS-induced NO production. The RAW264.7 cells were incubated in a medium containing 4-hydroxykobusin (3, 10, 30, 100 μM) for 10 min and then treated with LPS at 1 μg/ml. The amount of nitrite in the medium was monitored for 48 h. (C) Effects of 2-, 8--biapigenin on LPS-induced NO production. Raw264.7 cells were incubated in a medium containing 2-, 8--biapigenin (3, 10, 30 or 100μM) for 10 min and then treated with LPS 1μg/ml. The amount of nitrite in medium was monitored for 48 h. Data represents means_±_S.D. of 4 different samples (significant compared to LPS alone, ≤*p*-0.01).

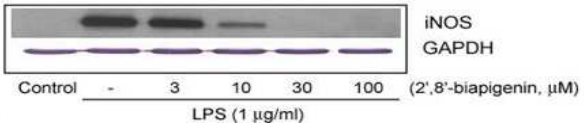
A)

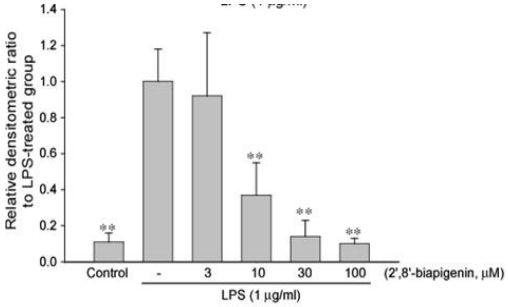

iNOS
GAPDH
Control - 3 10 30 100 (Taiwaniaflavone, μM)
Relative densitometric ratio to LPS-treated group
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control - 3 10 30 100 (Taiwaniaflavone, mM)
LPS (1 mg/ml)

B)

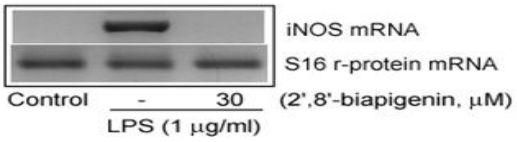

iNOS
GAPDH
Control - 3 10 30 100 (4-hydroxykobusin μM)
LPS (1 mg/ml)
LPS (1 mg/ml)
Relative densitometric ratio to LPS-treated group
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
**
Control - 3 10 30 100 (4-hydroxykobusin, mM)
LPS (1 mg/ml)

C)


iNOS
GAPDH
Control - 3 10 30 100 (2',8'-biapigenin, μM)
LPS (1 μg/ml)


Relative densitometric ratio to LPS-treated group
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control - 3 10 30 100 (2',8'-biapigenin, μM)
LPS (1 μg/ml)

D)


iNOS mRNA
S16 r-protein mRNA
Control - 30 (2',8'-biapigenin, μM)
LPS (1 μg/ml)

E)

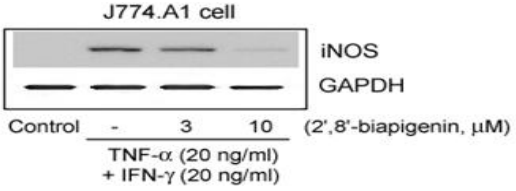

J774.A1 cell
iNOS
GAPDH
Control - 3 10 (2',8'-biapigenin, μM)
TNF-α (20 ng/ml)
+ IFN-γ (20 ng/ml)

Fig. 10. **Effects of Natural compounds on iNOS expression**. (A) Inhibition of LPS-inducible iNOS protein expression by taiwaniaflavone (3–100 μM). iNOS protein levels were monitored for 12 h after treating cells with LPS (1μg/ml) with or without taiwaniaXavone treatment. Relative iNOS protein levels were determined by measuring immunoblot band intensities by scanning densitometry. (B) Inhibition of LPS-inducible iNOS protein expression by 4-hydroxykobusin (3–100 μM). The level of iNOS protein was monitored 18 h after treating cells with LPS (1μg/ml) with or without 4-hydroxykobusin. Relative iNOS protein levels were determined by measuring immunoblot band intensities by scanning densitometry. Inhibition of LPS-inducible (C) iNOS protein expression by 2-, 8--biapigenin (3-100 μM). Levels of iNOS protein were monitored 12 h after treating cells with LPS (1m g/ml) with/without 2-,8--biapigenin treatment. Relative iNOS protein levels were determined by measuring immunoblot band intensities by scanning densitometry.(D)Inhibition of LPS-inducible iNOS mRNA expression by 2-, 8--biapigenin. iNOS mRNA expression levels were determined by RT-PCR. S16 ribosomal protein mRNA expression was comparable in samples. (E) Inhibition of tumor necrosis factor-a (TNF-α) and interferon- g (IFN-γ)-inducible iNOS protein expression by 2-,8-biapigenin (3, 10m M) in J774.A1 cells. Levels of iNOS protein were monitored 12 h after treating cells with TNF-a (20 ng/ml) and IFN-g (20 ng/ml) with/without 2-, 8--biapigenin treatment.

7. 2. *Taiwaniaflavone,* 4-hydroxykobusin, 2', 8"-biapigenin inhibits LPS-inducible NF-κB and AP-1 activation

To determine wheather the process of iNOS gene transcription is targeted by taiwaniaflavone, reporter gene analysis was performed using macrophages transfected with the mammalian cell expression vector pGL-miNOS1588, which contained luciferase cDNA and a-1.59 kb miNOS promoter (Woo et al., 2005) LPS (1 μg/ml) increased the luciferase activity by approximately 3.2 fold, and this enhanced activity was reversed by taiwaniaflavone at 10 or 30 μM (Fig. 11A). NF-κB and AP-1 are activated in cells stimulated with LPS or by some other inflammatory insult, which is involved in the transcriptional activation of responsive genes (Muller et al., 1993; Adcock et al., 1997; Guha et al., 2001). Hence, we carried out reporter gene analyses using luciferase reporter plasmids containing the NF-κB or AP-1 binding sequences to determine whether the suppressive effect of taiwaniaflavone on *iNOS* gene induction is with the inhibition of NF-κB or AP-1. LPS treatment (1 μg/ml, 18 h) caused a 5.6-fold increase in NF-κB reporter activity (Fig. 11B), and pretreatment of cells with 10 or 30 μM of taiwaniaflavone

significantly inhibited the increase in NF-kB reporter activity by LPS (Fig. 11B). However, LPS-induced AP-1 reporter activity increases were not significantly altered by taiwaniaflavone (Fig. 11C). These results demonstrate that taiwaniaflavone selectively inhibits the NF-κB activation process, and suggest that this is associated with an abrogation of iNOS induction by taiwaniaflavone.

Because p65 is a major participant in NF-κB activation by LPS in macrophages, we also examined p65 translocation to the nucleus by subcellular fractionation and immunoblotting. Nuclear p65 protein levels increased from 30min to 1h after treating RAW264.7 cells with LPS (1 μg/ml) and peaked at 30 min after LPS treatment. 30 μM taiwaniaflavone completely blocked the LPS-induced nuclear translocation of p65 at 1 h of treatment (Fig. 12A). This translocation is preceded by the phosphorylation and subsequent degradation of the I-κB subunit (Wang et al., 2002), and thus, we further examined phosphorylated I-κB and total I-κB levels in macrophages. Immunoblot analysis using specific antibodies showed that the phosphorylation and degradation of I-κB by LPS (1 μg/ml, 15 min) were also prevented by pretreating with 30 μM taiwaniaflavone (Fig. 12B). Phosphorylation step of I-κB is

dependent on the activation of IKK complex and the phosphorylation of IKK is essentially required for the activation of IKK (Zandi et al., 1997). Thus, we also determined phosphorylation levels of IKK/IKK in LPS-treated RAW264.7 cells. LPS resulted in transient phosphorylation of IKK/IKK (5 min) and this increase was completely inhibited by taiwaniaflavone pretreatment (Fig. 12B). These results provide evidence that the inhibition of NF-κB activation by taiwaniaflavone is due to the prevention of IKK complex activation and of I-κB phosphorylation.

To determine whether the process of *iNOS* gene transcription is affected by 4-hydroxykobusin, reporter gene analysis was performed using RAW264.7 cells transfected with the mammalian cell expression vector pGL-miNOS1588, which contained luciferase cDNA and a –1.6 kb miNOS promoter. LPS (1 μg/ml) increased the luciferase activity, and this increase was reversed by 4-hydroxykobusin at 30 or 100 μM (Fig. 13A).

One of key transcription factors involved in the transcription of *iNOS* gene is AP-1(Cho et al., 2002; Chen et al., 2003; Choi et al., 2005). AP-1 activation was assessed by reporter gene assay using luciferase plasmid containing AP-1 minimal promoter. The increase

in AP-1 reporter activity by LPS was 51% inhibited by 100 μM 4-hydroxykobusin (Fig. 13B), but 30 μM 4-hydroxykobusin did not suppress AP-1 reporter activity enhancement by LPS (1 μg/ml) (Fig. 13B). These findings suggest that blocking of AP-1 activation may be partly associated with the mechanism of iNOS expressional inhibition by 4-hydroxykobusin.

Inhibition of iNOS expression by 4-hydroxykobusin may result from the suppression of NF-κB activation. First, we performed reporter gene assay using a luciferase plasmid containing NF-κB minimal promoter. LPS treatment (1 μg/ml, 18 h) caused a 3-fold increase in NF-κB reporter activity (Fig. 13C), and pretreatment of cells with 100 μM of 4-hydroxykobusin completely inhibited the increase in NF-κB reporter activity by LPS (Fig. 13C).

We measured nuclear p65 levels by subcellular fractionation and immunoblotting. Nuclear p65 protein levels increased from 15 min to 30 min after treating RAW264.7 cells with LPS (1 μg/ml) and peaked at 30 min after LPS treatment. Pretreatment of cells with 100 μM 4-hydroxykobusin for 10 min suppressed the LPS-induced nuclear translocation of p65 (Fig. 14A). We further examined phosphorylated I-κB. levels in macrophages. Immunoblot analysis

using specific antibodies revealed that the I-κB phosphorylation by LPS (1 μg/ml) were also reversed by 100 μM 4-hydroxykobusin (Fig. 14B). These results indicate that the inhibition of NF-κB activation by 4-hydroxykobusin is due to the prevention of I-κB phosphorylation and the subsequent nuclear translocation of p65.

We additionally assessed the effect of 4-hydroxykobusin on the LPS-inducible COX-2 expression. COX-2 expression was not altered by up to 100 μM 4-hydroxykobusin (Fig. 14D). The expression of both *iNOS* and *COX-2* genes is dependent on NF-κB activation (Schmedtje et al., 1997; Newton et al., 1997). However, other cis-acting elements such as C/EBP binding site and cAMP response element (CRE) are also involved in the transcriptional regulation of the *COX-2* gene (Billack et al., 2002; Gorgoni et al., 2001). Although NF-κB binding in the promoter region of *COX-2* gene can be blocked by the lignan, other cis-acting elements may be still active. Hence, the minimal effect of 4-hydroxykobusin on COX-2 expression may result from the discrepancy of active transcription factors between *iNOS* and *COX-2* genes.

To confirm whether the process of iNOS gene transcription is targeted by 2', 8"-biapigenin, reporter gene assays

were performed using macrophages transfected with a mammalian cell expression vector pGL-miNOS1588, which contained the luciferase structural gene and a -1.59 kb miNOS promoter (Woo et al., 2005) 1 μg/ml LPS caused a 3.8-fold increase in luciferase activity and the enhanced reporter activity was reversed by 10 or 30 μM 2', 8"-biapigenin (Fig. 15A).

We performed reporter gene analysis using a luciferase plasmid containing the NF-κB binding sequence to determine whether the transcriptional inhibition of the iNOS gene by 2', 8"-biapigenin is related to NF-κB activation. LPS treatment (18 h) caused a 4.2-fold increase in NF-κB reporter activity (Fig. 15B), and the pretreatment of cells with 10 or 30 μM of 2', 8"-biapigenin significantly suppressed LPS-inducible NF-κB reporter activity (Fig. 15B).

Because p65 is a major component of NF-κB activation by LPS in macrophages, we also examined p65 translocation into the nucleus by subcellular fractionation and immunoblot analysis. Nuclear p65 protein levels were increased from 30 min to 1 h after treating Raw264.7 cells with LPS (1 μg/ml) and peaked 30 min after LPS treatment. However, pretreatment with 2', 8"-biapigenin (30

μM) almost completely blocked the LPS-induced nuclear translocation of p65 (Fig. 15C). These data show that the inhibition of iNOS gene expression by 2', 8"-biapigenin was due to the transcriptional inhibition of the iNOS gene through NF-κB pathway.

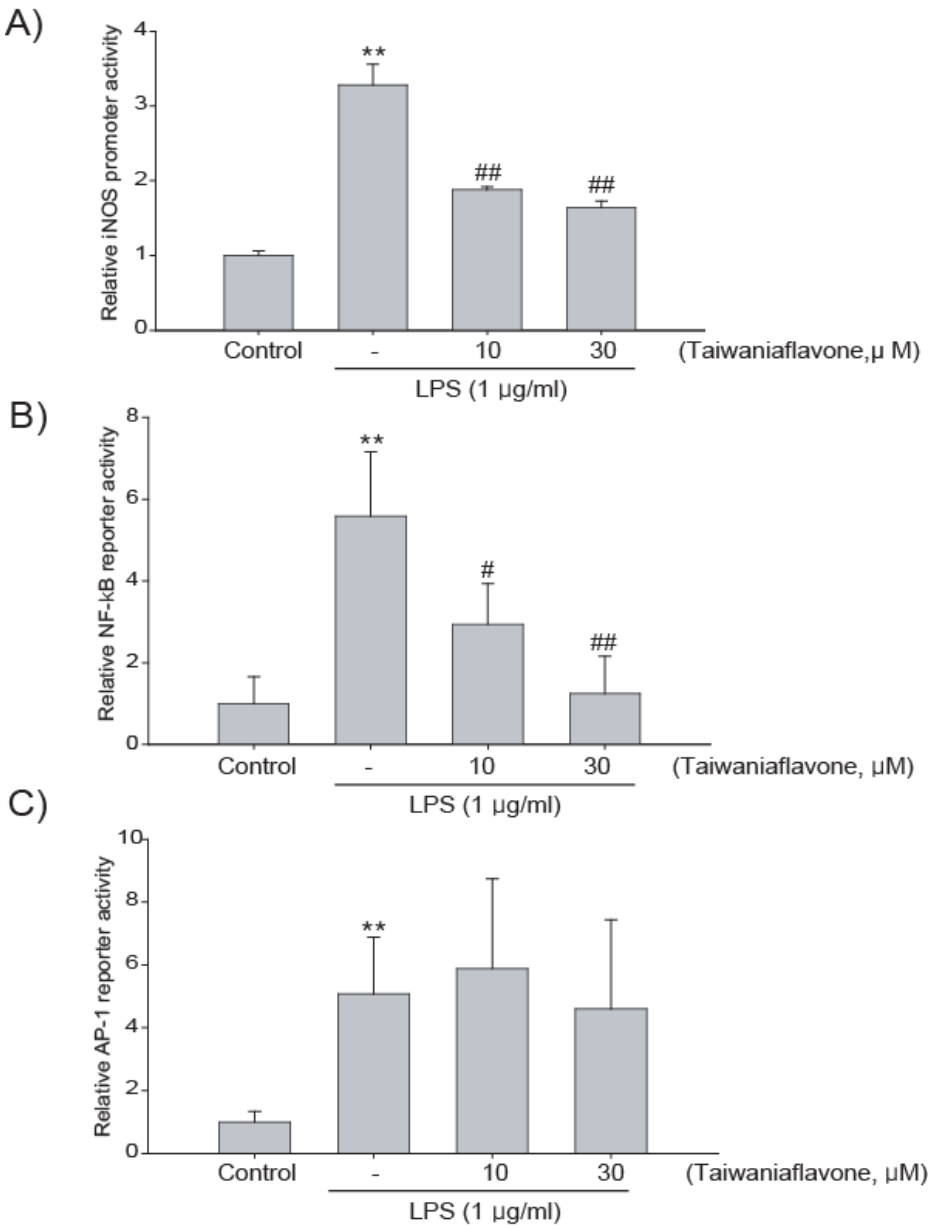
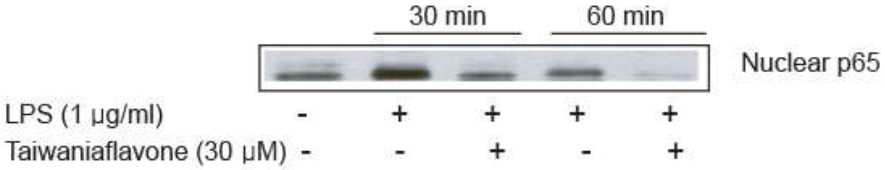

A)
Relative iNOS promoter activity
0
1
2
3
4
**
##
##
Control
-
10
30
(Taiwaniaflavone,μ M)
LPS (1 μg/ml)
B)
Relative NF-κB reporter activity
0
2
4
6
8
**
#
##
Control
-
10
30
(Taiwaniaflavone, μM)
LPS (1 μg/ml)
C)
Relative AP-1 reporter activity
0
2
4
6
8
10
**
Control
-
10
30
(Taiwaniaflavone, μM)
LPS (1 μg/ml)

Fig. 11**. Effect of taiwaniaflavone on LPS-induced NF-κB activation.** (A) Inhibitory effect of taiwaniaflavone on *iNOS* gene transactivation. Induction of luciferase activity by LPS in RAW264.7 cells transiently transfected with pGL-miNOS1588 construct, which contained a 1588 bp iNOS promoter sequence, was confirmed using a luminometer. A dual luciferase reporter gene assay was performed on lysed cells co-transfected with pGL-miNOS1588 (firefly luciferase) and pRL-SV (*Renilla* luciferase)(in the ratio of 50:1) after exposure to LPS (1μg/ml) and taiwaniaflavone (10 and 30 μM) for 18 h. Reporter gene activations were expressed as changes relative to *Renilla* luciferase activity. The results shown represent the means§ SD of four separate experiments (significant versus the control, ¤¤$p < 0.01$; signiWcant versus the LPS-treated group, ##$p < 0.01$). (B) NF-κB reporter gene analysis. Cells were transfected with the pNF-κB-Luc plasmid, and reporter gene analysis was performed as described in (A). Data represent the means §SD of 4 separate experiments (significant versus the control, ¤¤$p < 0.01$; significant versus the LPS-treated group, #$p < 0.05$, ##$p < 0.01$). (C) AP-1 reporter gene analysis. Cells were transfected with pAP-1-Luc plasmid, and reporter gene analysis was performed as described in (A). Results represent the means§ SD of four separate experiments (significant versus the control, ¤¤$p < 0.01$).

A)


30 min
60 min
Nuclear p65
LPS (1 μg/ml) - + + + +
Taiwaniaflavone (30 μM) - - + - +

B)

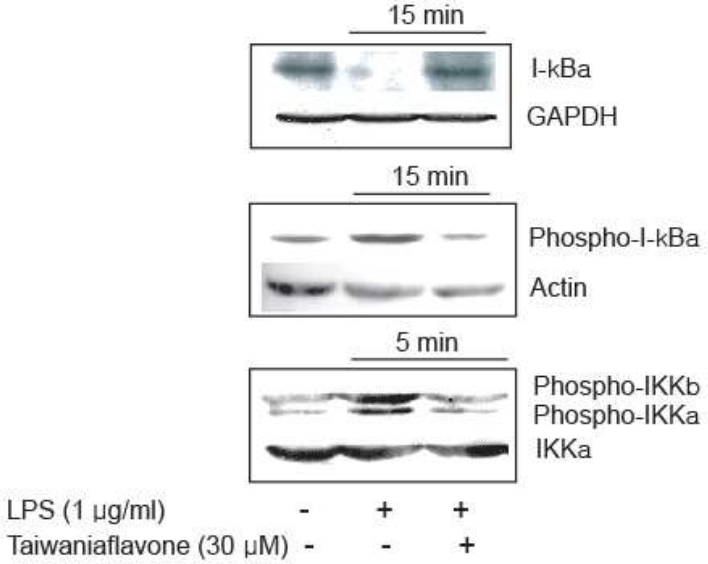
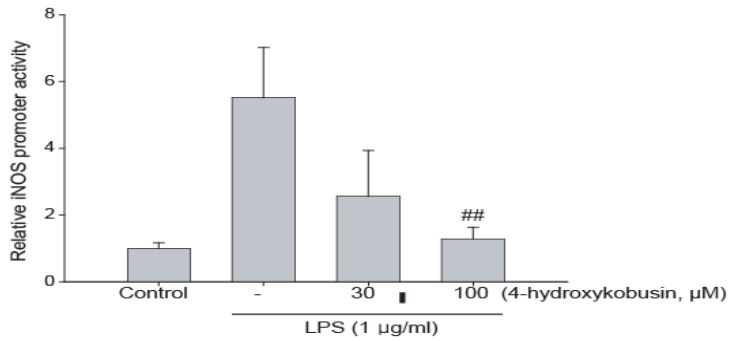
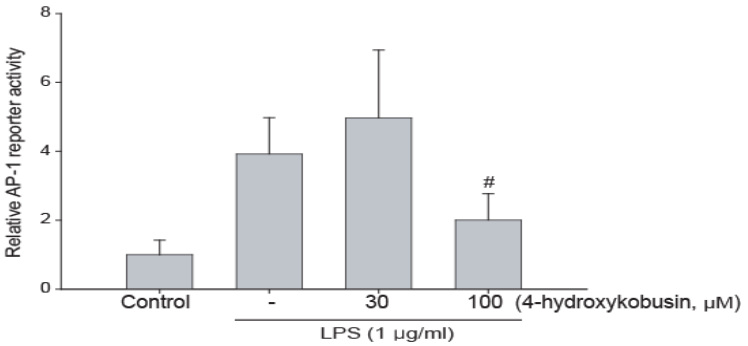

15 min
I-kBa
GAPDH
15 min
Phospho-I-kBa
Actin
5 min
Phospho-IKKb
Phospho-IKKa
IKKa
LPS (1 μg/ml) - + +
Taiwaniaflavone (30 μM) - - +

Fig. 12. (A) **Effects of taiwaniaflavone on p65 nuclear translocation**. RAW264.7 cells were treated with 1 μg/ml LPS for 30min or for 60 min with/without 30 μM taiwaniaflavone, and the protein levels of nuclear p65 was determined immunochemically using specific antibody. (B) Effects of taiwaniaflavone on the phosphorylation/degradation of I-κBα and on the phosphorylation of IKKα/ β. To determine I-κBα levels, cell lysates were obtained 15 min after exposure of RAW264.7 cells to LPS (1 μg/ml). GAPDH levels were measured with duplicate blot using same samples. The levels of phosphorylated I-κBα and phosphorylated IKKα/IKK β were determined using specific antibodies. The levels of actin and IKKα were measured as internal loading controls.

A)


Relative iNOS promoter activity
8
6
4
2
0
##
Control
-
30
100
(4-hydroxykobusin, μM)
LPS (1 μg/ml)

B)


Relative AP-1 reporter activity
8
6
4
2
0
#
Control
-
30
100
(4-hydroxykobusin, μM)
LPS (1 μg/ml)

C)

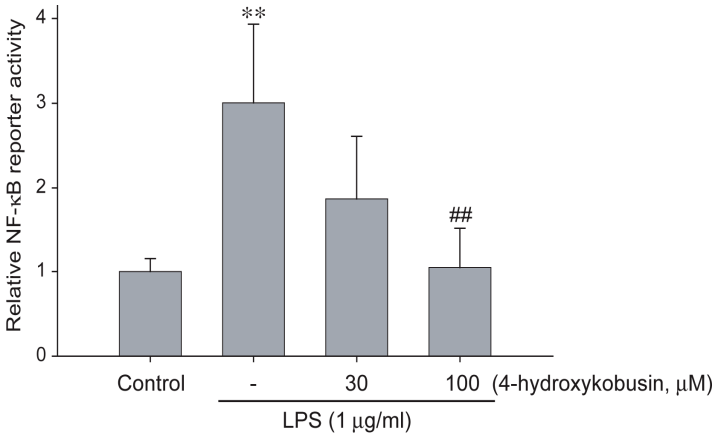
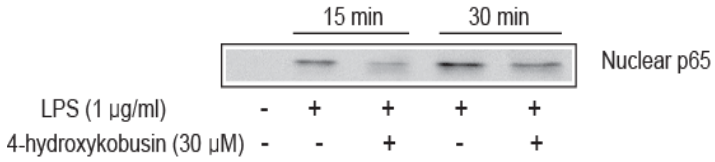

Relative NF-κB reporter activity
4
3
2
1
0
**
##
Control
-
30
100
(4-hydroxykobusin, μM)
LPS (1 μg/ml)

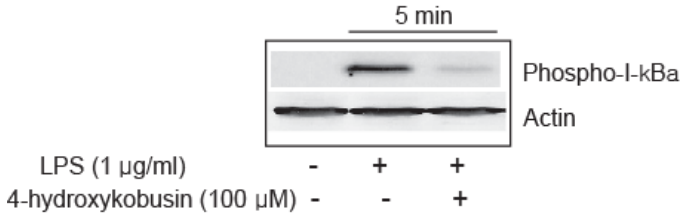
Fig. 13. **Effects of 4-Hydroxykobusin on the Transcriptional Activities of iNOS Promoter and AP-1 Minimal Promoter.** (A) Inhibitory effect of 4-hydroxykobusin on the transactivation of *iNOS* gene. Induction of luciferase activity by LPS in the RAW264.7 cells transiently transfected with pGL-miNOS1588 construct, which contained -1588 bp iNOS promoter sequences, was confirmed using a luminometer. A dual luciferase reporter gene assay was performed on the lysed cells co-transfected with pGL-miNOS1588 (firefly luciferase) and pRL-SV (*Renilla* luciferase) (in a ratio of 100:1) after exposure to LPS (1μg/ml) and 4-hydroxykobusin (30 and 100 μM) for 18 h. The cells were preincubated with 4-hydroxykobusin for 10 min before LPS exposure. The activation of the reporter gene was calculated as a relative change in the *Renilla* luciferase activity. Data represents the means ± SD of 3 separate samples (significant versus the control, $^{**}p<0.01$; significant versus the LPS-treated group, $^{**}p<0.01$). (B) AP-1 reporter gene analysis. Cells were transfected with the pAP-1-Luc plasmid, and reporter gene analysis was performed as described in panel (A) the data shown is representative of the means ± SD of 5 separate samples (significant versus the control, $^{**}p<0.01$; significant versus the LPS-treated group, $^{\#}p<0.05$).

(C) Cells were transfected with the pNF-kB-Luc plasmid, and reporter gene analysis was performed as described in the legend of (A). Data represents the means_±S.D. of 4 separate samples (significant *versus* the control, ***p*<0.01; significant *versus* the LPS-treated group, ** *p*<0.01).

A)


15 min
30 min
Nuclear p65
LPS (1 μg/ml) - + + + +
4-hydroxykobusin (30 μM) - - + - +

B)


5 min
Phospho-I-kBa
Actin
LPS (1 μg/ml) - + +
4-hydroxykobusin (100 μM) - - +

C)

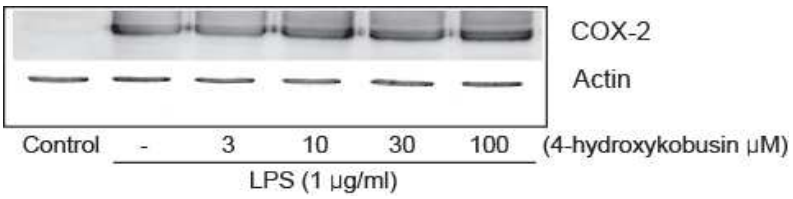

COX-2
Actin
Control - 3 10 30 100 (4-hydroxykobusin μM)
LPS (1 μg/ml)

Fig. 14.Effect of 4-Hydroxykobusin on the LPS-Inducible NF-kB Activation.(A) Effect of 4-hydroxykobusin on the LPS-induced nuclear translocation of p65. RAW264.7 cells were treated with 1 μg/ml of LPS for 15 min or 30 min in the presence or absence of 100 μM 4-hydroxykobusin, and nuclear p65 protein was immunochemically detected using antip65 antibody. (B) Effect of 4-hydroxykobusin on LPS-inducible I-kBa phosphorylation. The phosphorylation of I-kBa was immunochemically assessed 5 min after 1 μg/ml LPS exposure to RAW264.7 cells. The cells were preincubated with 100 μM 4- hydroxykobusin for 10 min. (C) Effect of 4-hydroxykobusin (3-100 μM) on COX-2 expression. COX-2 protein levels were monitored 18 h after treating cells with LPS (1 μg/ml).

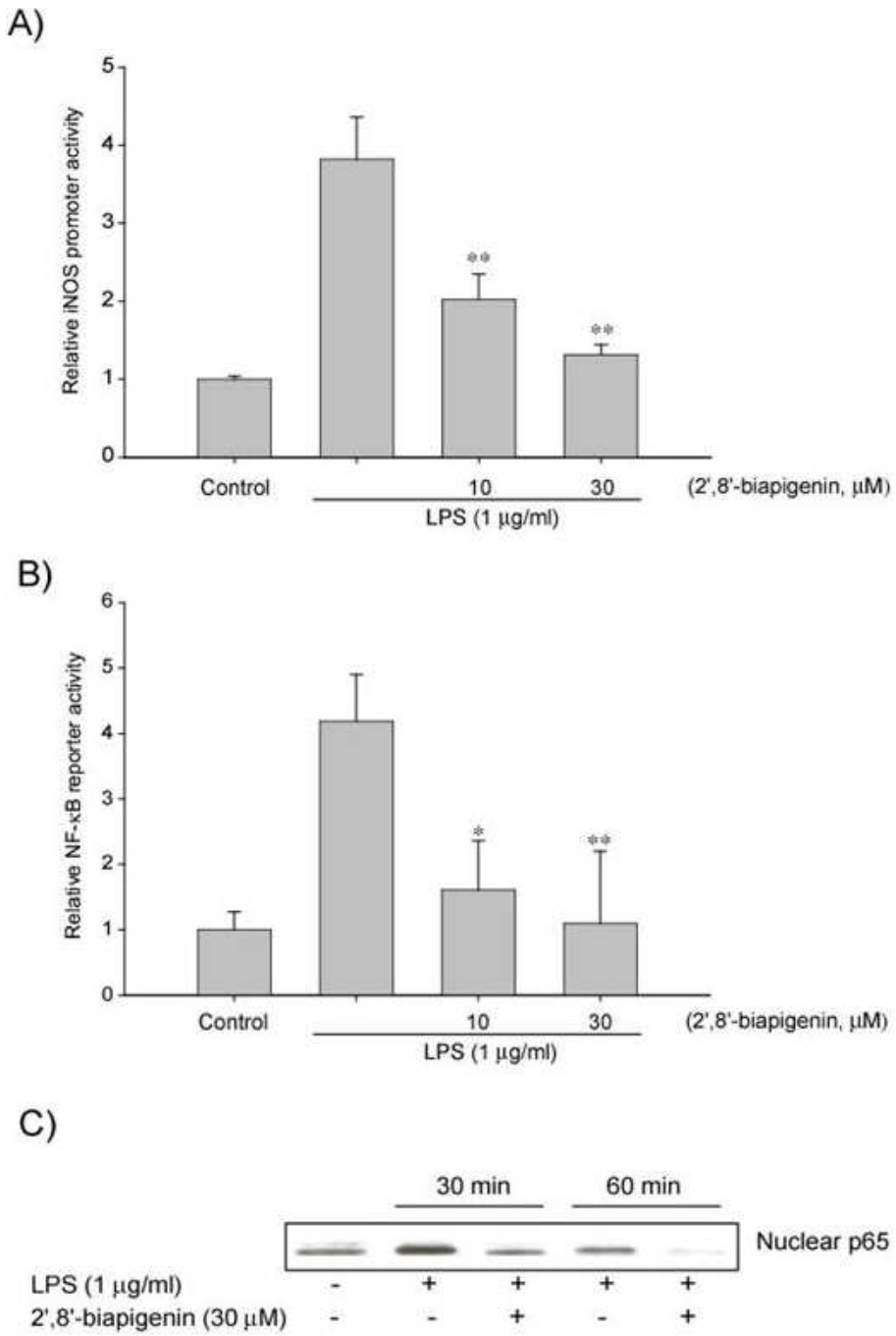

A)
Relative iNOS promoter activity
0
1
2
3
4
5
**
**
Control
10
30
(2',8'-biapigenin, μM)
LPS (1 μg/ml)
B)
Relative NF-κB reporter activity
0
1
2
3
4
5
6
*
**
Control
10
30
(2',8'-biapigenin, μM)
LPS (1 μg/ml)
C)
30 min
60 min
Nuclear p65
LPS (1 μg/ml)
- + + + +
2',8'-biapigenin (30 μM)
- - + - +

Fig. 15. Effects of 2', 8"-biapigenin on LPS-inducible NF-κB activation. (A) Inhibitory effect of 2', 8"-biapigenin on iNOS gene transactivation. Induction of luciferase activity by LPS in Raw264.7 cells transiently transfected with a pGL-miNOS1588 construct containing -1588 bp iNOS promoter sequences, were confirmed using a luminometer. A dual luciferase reporter gene assay was performed on lysed cells co-transfected with pGL-miNOS1588 (firefly luciferase) and pRL-SV (*Renilla* luciferase) (in a ratio of 50:1) after exposure to LPS (1 μg/ml) and 2', 8"-biapigenin (10 and 30 μM) for 18 h. Reporter gene activation is expressed versus *Renilla* luciferase activity. Data represents the means ± SD of 4 separate experiments (significant versus the control, $^{**}p<0.01$; significant versus the LPS-treated group, $^{\#\#}p<0.01$). (B) NF-κB reporter gene analysis. Cells were transfected with pNF-κB-Luc plasmid, and reporter gene analysis was performed as described in panel (A). Data represent the means ± SD of 4 separate experiments (significant versus the control, $^{**}p<0.01$; significant versus the LPS-treated group, $^{\#}p<0.05$, $^{\#\#}p<0.01$). (C) Effect of 2', 8"-biapigenin on p65 nuclear translocation. Raw264.7 cells were treated with 1 μg/ml LPS for 30 min or 1h with or without 30 μM 2', 8"-biapigenin, and

nuclear p65 protein was immunochemically detected using anti-p65 antibody.

7. 3. Inhibition of COX-2 induction and PGE2 production by *Taiwaniaflavone*, 4-hydroxykobusin and 2', 8"-biapigenin

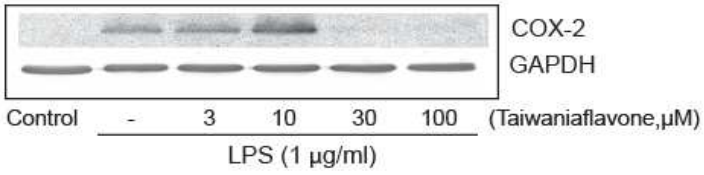
There are two NF-κB consensus sequences in the promoter region of the COX-2 gene, and COX-2 gene expression is dependent on NF-κB activation (Schmedtje et al., 1997; Newton et al., 1997). Thus, we also investigated whether taiwaniaflavone inhibits COX-2 induction and PGE2 synthesis in LPS-stimulated macrophages (Fig. 16A). Western blot analysis showed that exposure of cells to LPS (1 μg/ml) for 24 h increased COX-2 protein levels (Fig. 16A). Moreover, taiwaniaflavone at 30 or 100 μM significantly reduced COX-2 expression level (Fig. 16A). Since PGE2 is one of the stable autacoids produced by COX-2 in activated macrophages (Chen et al., 2003), we examined PGE2 levels in culture medium. When cells were exposed to 1 μg/ml LPS for 24 h, PGE2 levels increased. And, the enhanced PGE2 production was significantly diminished in cells pretreated with 30 or 100 μM taiwaniaflavone prior to LPS treatment (by 54 and 82 %, respectively) (Fig. 16B).

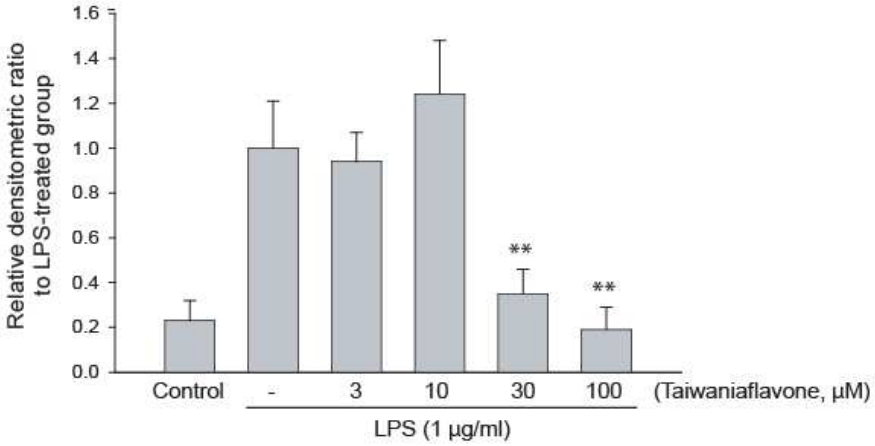
We investigated whether 2', 8"-biapigenin affects LPS-mediated PGE2 synthesis in macrophages (Fig. 17A). When cells

were exposed to 1 μg/ml LPS for 24 h, PGE2 levels showed about 4-fold increase compared with the controls. Moreover, this enhanced PGE2 production was significantly diminished in the cells pretreated with 100 μM 2′, 8″-biapigenin (68 % inhibition).

To determine whether the decreased PGE2 production by 2', 8"-biapigenin could be related with the expression of COX-2 protein, we performed Western blot analysis using COX-2 specific antibody. The stimulation of Raw264.7 cells with LPS for 24 h also induced the expression of COX-2 protein (Fig. 17B), and 100 μM 2', 8"-biapigenin significantly reduced COX-2 protein levels. However, low concentrations (3, 10 or 30 μM) of 2', 8"-biapigenin did not affect COX-2 expression (Fig.174B).

A)


COX-2
GAPDH
Control
-
3
10
30
100
(Taiwaniaflavone,μM)
LPS (1 μg/ml)


Relative densitometric ratio to LPS-treated group
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
Control
-
3
10
30
100
(Taiwaniaflavone, μM)
LPS (1 μg/ml)

B)

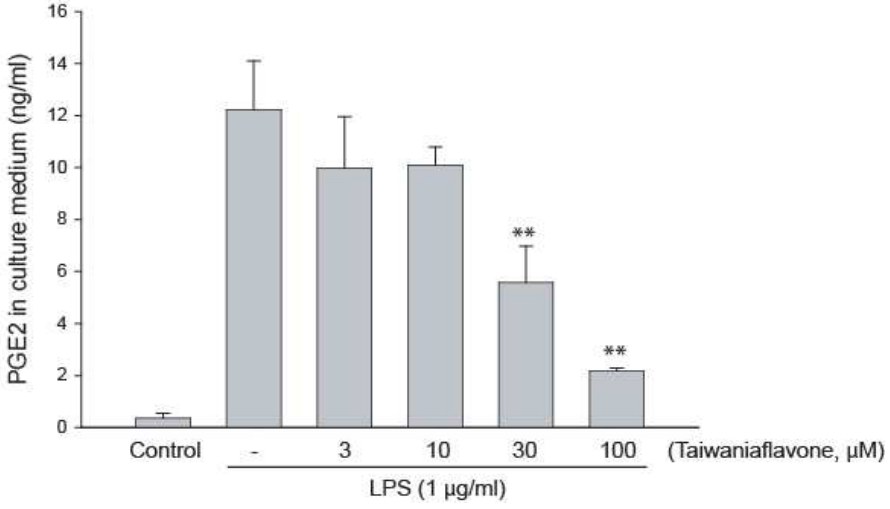
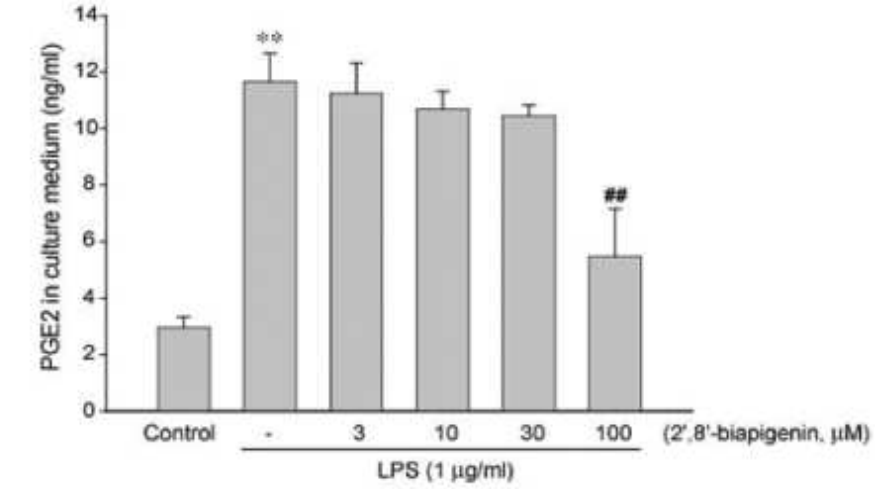
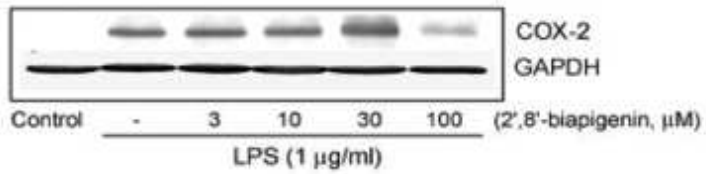

PGE2 in culture medium (ng/ml)
16
14
12
10
8
6
4
2
0
**
**
Control
-
3
10
30
100
(Taiwaniaflavone, μM)
LPS (1 μg/ml)

Fig. 16. Effect of taiwaniaflavone on COX-2 expression. (A) Inhibition of LPS-inducible COX-2 protein expression by taiwaniaflavone (3-100 μM). COX-2 protein levels were monitored 24 h after treating cells with LPS (1 μg/ml). Relative COX-2 protein levels were determined by measuring immunoblot band intensities by scanning densitometry. Data represent means±SD of three separate experiments (significant compared to LPS alone, $^{**}P<0.01$). (B) Effect of taiwaniaflavone on LPS-induced PGE2 production in macrophages. RAW264.7 cells were incubated with 1 μg/ml LPS for 24 h and amounts of PGE2 in medium was determined using PGE2-specific ELISA assays. The results shown represent the means±SD of 4 different samples.

A)


PGE2 in culture medium (ng/ml)
14
12
10
8
6
4
2
0
**
##
Control
-
3
10
30
100
(2',8'-biapigenin, μM)
LPS (1 μg/ml)

B)


COX-2
GAPDH
Control
-
3
10
30
100
(2',8'-biapigenin, μM)
LPS (1 μg/ml)

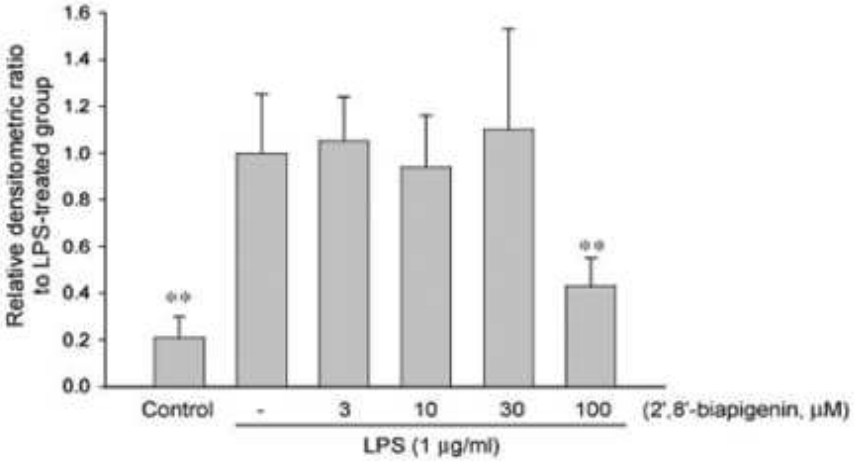

Relative densitometric ratio to LPS-treated group
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
Control
-
3
10
30
100
(2',8'-biapigenin, μM)
LPS (1 μg/ml)

Fig. 17. Effects of 2', 8"-biapigenin on COX-2 expression. (A) Effects of 2', 8"-biapigenin on LPS-induced PGE2 production in macrophages. Raw264.7 cells were incubated with 1 μg/ml LPS for 24 h and the amount of PGE2 in medium was determined by PGE2-specific ELISA. Data represent the means ± SD of 4 different samples (significant compared to control group, $^{**}p<0.01$; significant compared to LPS alone, $^{\#\#}p<0.01$). (B) Inhibition of LPS-inducible COX-2 protein expression by 2', 8"-biapigenin (3 – 100 μM). COX-2 protein levels were monitored 24 h after treating cells with LPS (1 μg/ml). Relative COX-2 protein levels were determined by measuring immunoblot band intensities by scanning densitometry. Data represent the means ± SD of three separate experiments (significant compared to LPS alone, $^{**}p<0.01$).

8. Discussion of Phytochemiclas' therapeutics.

The productions of proinflammatory cytokines, prostaglandins, and NO by activated macrophages play critical roles in severe inflammatory diseases such as sepsis and arthritis (Szabo et al., 1998; Martel-Pelletier et al., 2003). Hence, the inhibition of proinflammatory cytokines and iNOS/COX-2 gene expression in inflammatory cells, such as macrophages, may offer a new therapeutic strategy against inflammation. As a result of our on-going screening of anti-inflammatory agents from plants, we became interested in the effect of taiwaniaflavone and 2', 8"-biapigenin on NF-κB- mediated iNOS/ COX-2 expression induced by LPS. In the same way lignan, 4-hydroxykobusin from *Geranium thunbergii* inhibits iNOS expression in macrophages, suggesting that 4-hydroxykobusin is a naturally-occurring iNOS inhibitor. We showed that IC_{50} value of 4-hydroxykobusin on the nitrite production is ~30

M. Park et al. recently reported that a lignan, lappaol F isolated from *Arctium lappa* more potently inhibited NO production (IC_{50} = 9.5 M) in comparison to 4-hydroxykobusin (Park et al., 2007). The potency discrepancy between lappaol F and 4-hydroxykobusin would be due to the structure difference. Because lappaol F is classified as a

di-lignan, lappaol F may metabolized to two different compounds containing active lignan moiety.

In the present study, we found that taiwaniaflavone inhibits the activities of inducible forms of COX and NOS in macrophages, and that taiwaniaflavone and2', 8"-biapigenin probably act at the transcriptional level. A reporter gene analysis using iNOS promoter, showed that the LPS-inducible transactivation of the iNOS gene was significantly suppressed by taiwaniaflavone, and 2', 8"-biapigenin thus indicating these natural compounds target the transcription step. The promoter regions of the iNOS and COX-2 genes contain NF-κB binding sites (Schmedtje et al., 1997; Xie et al., 1993). Moreover, NF-κB is known to be an essential transcription regulator of these two genes (Diaz-Guerra et al., 1996; Lee et al., 2003). Hence, by using a reporter gene assay using NF-κB minimal promoter and the immunochemical detection of nuclear p65, we found that taiwaniaflavone2', and 8"-biapigenin potently suppresses NF-κB activity. We further revealed that 4-hydroxykobusin mainly acted on the transcriptional process of *iNOS* gene. A reporter gene analysis using *iNOS* promoter, showed that the LPS-inducible transactivation of the *iNOS* gene was significantly suppressed by 4-hydroxykobusin,

thus indicating the lignan targets the transcription of *iNOS* gene.

The NF-κB heterodimer of p65 and p50, is located in the cytoplasm as an inactive complex bound to I-κB, which is phosphorylated and subsequently degraded, and then dissociates to produce activated NF-κB. In the present study, we found that the phosphorylation and degradation of I-κB, which are required for p65 activation, were abolished in cells treated with taiwaniaflavone. The phosphorylation of I-κB bound to NF-κB is considered to be mediated by IKK at two conserved serines within the N-terminal domain of I-κB. Karin et al., 2000). And, it has been reported that some natural flavonoids suppress the activity of I-κ/B kinase (Pan et al., 2000; Yang et al., 2001), for example a green tea polyphenol, epigallocatechin-3-gallate, was found to directly block I-κB kinase activity in an intestinal epithelial cell line (Yang et al., 2001). In this study, we found that phosphorylation of IKK/IKK was also blocked by taiwaniaflavone. Since IKK complex can be activated by a variety of upstream kinases such as NF-κB-inducing kinase, protein kinase C and the tyrosine kinase family (Hayden et al., 2004; Huang et al., 2003; Trushin et al., 2003), taiwaniaflavone may also act on the upstream kinases of IKK complex and the exact molecular target(s)

affected by taiwaniaflavone remains to be identified.

Recent *in vitro* and *in vivo* studies have suggested that the inhibition of COX-2 and iNOS enzymes has an anti-carcinogenic effect (Watanabe et al., 2000; Lynch et al., 2001). Chinese herbs including *Sellaginella* genus plants have been used as alternative anti-cancer agents in East Asia (Lee et al., 1999; Mori et al., 1989). Hence, the present data provide a possible mechanistic basis for the anti-tumor or chemopreventive effect of *Sellaginella* extracts. The productions of excessive proinflammatory mediators, prostaglandins, and NO, through NF-κB pathway play an important role in severe inflammatory disease (Southan et al., 1989), and thus the inhibition of proinflammatory mediators in inflammatory cells could beneficially suppress excessive inflammatory reaction. In this study, we also found that 2', 8"-biapigenin inhibited the LPS-induced production of nitric oxide and PGE2 in macrophages by blocking NF-κB activation. Thus, the inhibition of this transcription factor by 2', 8"-biapigenin or *Sellaginella* extracts offers a possible therapeutic approach to the treatment of severe inflammatory diseases.

The *iNOS* gene promoter contains several homologous consensus sequences as binding sites for transcription factors

including NF-κB and AP-1 (Cieslik et al., 2002; Lee et al., 2003). Since NF- B and AP-1 are believed to be essentially required for *iNOS* gene transcription, (Cho et al., 2002; Xie et al., 1993; Diaz-Guerra et al., 1996), we performed reporter gene analyses using NF-κB and AP-1 minimal promoters. Reporter gene assays showed that 4-hydroxykobusin inhibited activation process of both NF-κB and AP-1. We further found that 4-hydroxykobusin completely blocked the nuclear translocation of p65 and the lignan was effective at blocking the phosphorylation of I-κB protein. These results combined with the data from NF-κB reporter gene assays suggested that the phosphorylation of I-κB is a pharmacological target of 4-hydroxykobusin. Since I-κB is serially phosphorylated by diverse upstream kinases such as I-κB kinase, NF-κB-inducing kinase, protein kinase C and the tyrosine kinase family, (Hayden et al.,2004; Huang et al., 2003; Trushin et al., 2003) the possible molecular target(s) of the lignan for the blocking of NF-κB seem to be one of the upstream kinases. It has been reported that a dibenzylbutyrolactone lignan, arctigenin concomitantly inhibits the activation of NF-κB and AP-1 in LPS-treated macrophages (Cho et al., 2002, 2004). In this study, we also showed that 4-hydroxykobusin

acted on the activation of both NF-κB and AP-1. Cho et al. revealed that MAP kinases and their upstream kinases MKK1 were inhibited by arctigenin and suggested that AP-1 inhibition by arctigenin resulted from its kinase blocking activity Cho et al., 2004). Therefore, the inhibitory effect of 4-hydroxykobusin on AP-1 activity also may be related with its actions on the upstream kinase(s) regulating MAP kinases. The exact molecular target(s) affected by 4-hydroxykobusin remains to be identified.

We recently showed that 7, 7'-dihydroxybursehernin from *Geranium thunbergii* inhibited LPS-inducible iNOS expression (Pokharel et al., 2007). The inhibitory potency of 4-hydroxykobusin against iNOS induction was very similar to that of 7, 7'-dihydroxybursehernin (Complete inhibition was seen in 100 M of both the lignans). However, the mechanism of iNOS inhibitory action by 7, 7'-dihydroxybursehernin is distinct from that by 4-hydroxykobusin. The pharmacological target of 7, 7'-dihydroxybursehernin is physical binding of NF-κB to DNA. Thus, both the lignans in *Geranium thunbergii* have identical functions to control transcription of *iNOS* gene, but their mechanistic bases would be different. It was also found that iNOS inhibitory activity of

kobusin was almost comparable to that of 4-hydroxykobusin (data not shown). Hence, the existence of hydroxyl group in 4-hydroxykobusin may not be critical to its NO blocking activity.

In this study, we found that tawaniaflavone was several times more potent to inhibit iNOS expression than COX2 expression. The expression of both iNOS and COX-2 genes is dependent on NF-κB activation (Schmedtje et al., 1997; Newton et al., 1997). However, other cis-acting elements such as C/EBP binding site, CRE and NF-IL6 site are also involved in the transcriptional regulation of the COX-2 gene (Billack et al., 2002; Gorgoni et al., 2001). Although NF- B-binding in the promoter region of COX-2 gene can be blocked by taiwaniaflavone, other cis-acting elements (e.g. CRE) may be still active.

NF-κB activation is controlled by cellular redox state (Gius et al., 1999; Kratsovnik et al., 2005). A variety of antioxidants such as ascorbic acid and tocopherol inhibit NF-κB (Tan et al., 2005) and oxygen-derived radicals are directly coupled with the NF-κB activation process (Brar et al., 2002). 2', 8"-biapigenin, a structurally similar bi-flavonoid, has an anti-oxidant function (Couladis et al., 2002), and it has also been reported that the bi-flavonoid

amentoflavone isolated from *S. tamariscina* directly scavenges superoxide anion (Huguet et al., 1990). Hence, the efficient blocking of NF-κB activation by 2', 8"-biapigenin might be associated with its potential antioxidant effects.

In summary, the present study, suggests that taiwaniaflavone isolated from *S. tamariscina* inhibits the expressions and activities of inducible isoforms of COX and NOS in macrophages. The potent COX-2 and iNOS inhibitory effects of taiwaniaflavone are associated with NF-κB inactivation via the blockade of I-κB phosphorylation. Since NF- B is one of the transcription factors that regulate the transcriptions of many genes associated with inflammation, inhibition of this transcription factor with taiwaniaflavone offers a possible approach to the treatment of severe inflammatory diseases.In the same way, 2', 8"-biapigenin inhibits the expression and activity of the inducible isoforms of COX and NOS in macrophages. This potent inhibitory effect of 2', 8"-biapigenin may be associated with the putative anti-inflammatory and anti-carcinogenic effects of *Sellaginella* extracts. The present study showed that 4-hydroxykobusin isolated from *G. thunbergii* inhibits the expressions and activities of inducible NOS in macrophages. The iNOS inhibitory

effects of 4-hydroxykobusin are associated with both NF-κB inactivation via the blockade of I-κB phosphorylation and AP-1 inactivation. Since NF- B and AP-1 are critical transcription factors that regulate the transcriptions of many genes associated with inflammation, inhibition of these transcription factors with 4-hydroxykobusin offers a possible approach to the treatment of severe inflammatory diseases

9. Conclusion.

1. We can conclude that, Pin1 is up-regulated in the chondrocytes, lymphocytes and fibroblasts of RA lesions of CII-injected RA mice and the Pin1 overexpression results in the induction of proinflammatory proteins including COX-2, iNOS, TNF-α and IL-1β. Pin1-dependent COX-2 expression is associated with the simultaneous activations of NF-κB, C/EBP and CREB.
2. Although RA is one of the most frequent inflammatory diseases, the molecular pathogenesis of this disease has not been totally clarified. Data presented here indicate that Pin1 is induced in the lesion area of CII-mediated arthritis and plays a key role in the excess production of proinflammatory mediators including Prostaglandins, NO, TNF-α and IL-1β. Several mechanisms may be involved in the overproduction of these multiple proteins in response to Pin1 overexpression. Pin1 may serve as a new therapeutic target of RA.
3. Juglone (a well known Pin1 inhibitor) treatments efficiently suppressed RA progress in CII-induced RA model and also inhibited the basal COX-2 expression in primary chondrocytes isolated from human RA patient. These results imply that Pin1

could be a potential pharmacological target of RA progress in clinics.

4. Taiwaniaflavone isolated from *S. tamariscina* inhibits the expressions and activities of inducible isoforms of COX and NOS in macrophages. The potent COX-2 and iNOS inhibitory effects of taiwaniaflavone are associated with NF-κB inactivation via the blockade of I-κBα phosphorylation. Since NF-κB is one of the transcription factors that regulate the transcriptions of many genes associated with inflammation, inhibition of this transcription factor with taiwaniaflavone offers a possible approach to the treatment of severe inflammatory diseases.
5. 2', 8"-biapigenin inhibits the expression and activity of the inducible isoforms of COX and NOS in macrophages. This potent inhibitory effect of 2', 8"-biapigenin may be associated with the putative anti-inflammatory and anti-carcinogenic effects of *Sellaginella* extracts.
6. 4-hydroxykobusin isolated from *G. thunbergii* inhibits the expressions and activities of inducible NOS in macrophages. The iNOS inhibitory effects of 4-hydroxykobusin are associated with both NF-κB inactivation via the blockade of I-κBα

phosphorylation and AP-1 inactivation. Since NF-κB and AP-1 are critical transcription factors that regulate the transcriptions of many genes associated with inflammation, inhibition of these transcription factors with 4-hydroxykobusin offers a possible approach to the treatment of severe inflammatory diseases.

10. References.

Adcock IM. Transcription factors as activators of gene transcription: AP-1 and NF-κB. Monald. Arch. Chest. Dis. 1997; (52) 178-186.

Alaaeddine N, Di Battista JA, Pelletier JP, et al. Inhibition of tumor necrosis factor alpha-induced prostaglandin E2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways. Arthritis Rheum. 1999; (42)710–718.

Alamanos Y, Voulgari PV, Drosos AA.Incidence and prevalence of rheumatoid arthritis, based on the 1987 American College of Rheumatology criteria: a systematic review.Semin Arthritis Rheum. 2006; 36(3):182-8.

Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Harrington AM, Shields PG, Felley-Bosco E, Hussain SP, Harris CC. Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. J. Natl. Cancer Inst. 1999; (91) 86-88.

Amin AR, Attur M, Patel RN, Thakker GD, Marshall PJ, Rediske J. Superinduction of cyclooxygenase-2 activity in human

osteoarthritis-affected cartilage: influence of nitric oxide. J Clin Invest 1997; 99:1231–7.

Anderson GD, Hauser SD, McGarity KL, Bremer ME, Isakson PC, Gregory SA. Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. J Clin Invest 1996;97:2672-9.

Arias-Negrete S, Keller K, Chadee K. Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophages, Biochem. Biophys. Res. Commun. 1995; (208) 582–589.

Balastik M, Lim J, Pastorino L, Lu KP. Pin1 in Alzheimer's disease: multiple substrates, one regulatory mechanism? Biochim Biophys Acta. 2007 Apr; 1772(4):422-9.

Bao L, Sauter G, Sowadski J, Lu KP, Wang D. Prevalent overexpression of prolyl isomerase Pin1 in human cancers. Am. J. Pathol. 2004; (164) 1727-1737.

Bayer E, Goettsch S, Mueller JW, Griewel B, Guiberman E, Mayr LM, Bayer P. Structural analysis of the mitotic regulator hPin1 in solution: insights into domain architecture and substrate binding. J. Biol. Chem. 2003; (278) 26183-26193.

Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol. 1991; 146: 108–13.

Bidgood MJ, Jamal OS, Cunningham AM, Brooks PM, Scott KF. Type IIA secretory phospholipase A2 up-regulates cyclooxygenase-2 and amplifies cytokine-mediated prostaglandin production in human rheumatoid synoviocytes. J. Immunol. 2000; 165(5):2790-7.

Billack B, Heck DE, Mariano TM, Gardner CR, Sur R, Laskin DL, Laskin JD. Induction of cyclooxygenase-2 by heat shock protein 60 in macrophages and endothelial cells. Am. J. Physiol. Cell. Physiol. 2002; (283) C1267-C1277.

Brahn E, Banquerigo ML, Firestein GS, Doyle DL, Salzman AL & Szaboá C. Collagen induced arthritis: reversal by mercaptoethylguanidine, a novel anti inflammatory agent with a combined mechanism of action. J. Rheumatol.1998b; (25) 1785 ± 1793.

Brar SS, Kennedy TP, Sturrock AB, Huecksteadt TP, Quinn MT, Murphy TM, Chitano P, Hoidal JR. NADPH oxidase promotes NF-κB activation and proliferation in human airway smooth muscle. Am. J. Physiol. Lung Cell. Mol. Physiol. 2002; (282)

L782-L795.

Bremner P, Heinrich M. Natural products as targeted modulators of the NF- κB pathway. J Pharm Pharmacol. 2002 Apr; 54(4):453-72.

Chen CW, Lee ST, Wu WT, Fu WM, Ho FM, Lin WW. Signal transduction for inhibition of inducible nitric oxide synthase and cyclooxygenase-2 induction by capsaicin and related analogs in macrophages, Br. J. Pharmacol. 2003; (140) 1077-1087.

Cheng JT, Chang SS, Hsu FL. Antihypertensive action of geraniin in rats. J. Pharm. Pharmacol. 1994; (46)46-49.

Chien SC, Liu HK, Kuo YH. Two new compounds from the leaves of *Calocedrus microlepic* var. formosana. Chem. Pharm. Bull. (Tokyo). 2004; (52) 762-763.

Cho MK, Jang YP, Kim YC, Kim SG. Arctigenin, a phenylpropanoid dibenzylbutyrolactone lignan, inhibits MAP kinases and AP-1 activation via potent MKK inhibition: the role in TNF-α inhibition. Int. Immunopharmacol. 2004 (4) 1419-1429.

Cho MK, Park JW, Jang YP, Kim YC, Kim SG. Potent inhibition of lipopolysaccharide-inducible nitric oxide synthase expression by dibenzylbutyrolactone lignans through inhibition of I-κBα phosphorylation and of p65 nuclear translocation in macrophages.

Int. Immunopharmacol. 2002 (2) 105-116.

Cho MK, Suh SH, Kim SG. JunB/AP-1 and NF-κB-mediated induction of nitric oxide synthase by bovine type I collagen in serum-stimulated murine macrophages. Nitric Oxide. 2002; (6) 319-332.

Choi DY, Lee JY, Kim MR, Woo ER, Kim YG, Kang KW. Chrysoeriol potently inhibits the induction of nitric oxide synthase by blocking AP-1 activation. J. Biomed. Sci. 2005; (12) 949-959.

Chung FL, Conaway CC, Rao CV, Reddy BS. Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. Carcinogenesis. 2000; (21) 2287-2291.

Cieslik K, Zhu Y, Wu KK. Salicylate Suppresses Macrophage Nitric-oxide Synthase-2 and Cyclo-oxygenase-2 Expression by Inhibiting CCAAT/Enhancer-binding Protein-β Binding via a Common Signaling Pathway. J. Biol. Chem. 2002; (277) 49304-49310.

Couladis M, Baziou P, Verykokidou E, Loukis A. Antioxidant activity of polyphenols from Hypericum triquetrifolium Turra. Phytother. Res. 2002; (16) 769-770.

Cuzzocrea S, Caputi AP, & Zingarelli B. Perox-ynitrite-mediated DNA strand breakage activates poly (ADP- ribose) synthetase and causes cellular energy depletion in carrageenan-induced pleurisy. Immunology. 1998b; (94) 93 ± 96.

Cuzzocrea S, Costantino G, Mazzon E, & Caputi AP. Bene®cial e€ects of raxofelast (IRFI 016), a new hydrophilic vitamin e-like antioxidant, in carrageenan-induced pleurisy. Br. J. Pharmacol. 1999a; (126) 407 ± 414.

Cuzzocrea S, Sautebin L, De Sarro GB, Costantino G, Rombolaá L, Mazzon E, Ialenti A, De Sarro A, Ciliberto G, Di Rosa M, Caputi AP, & Thiemermann C. Role of interleukin-6 in local in¯ammation. J. Immunol. 1999b ;(163) 5094 ± 5104.

Deborah PM Symmons. Epidemiology of rheumatoid arthritis: determinants onset, persistence and outcome. Best Practice and research clinical rheumatology. 2002; 16 (5), 707-722.

Deleuran BW, Chu CQ, Fieldm, Brennan FM, Katsiki P, Feldmann M, & Maini Rn. Localization of interleukin-1 a, type 1 interleukin-1 receptor and interleukin-1 receptor antagonist in the synovial membrane and cartilage/pannus junction in rheumatoid arthritis. Br. J. Rheumatol. 1992; 31,809.

Diaz-Guerra MJ, Velasco M, Martin-Sanz P, Bosca L. Evidence for Common Mechanisms in the Transcriptional Control of Type II Nitric Oxide Synthase in Isolated Hepatocytes. Requirement of NF-κB Activation after Stimulation with Bacterial Cell Wall Products and Phorbol Esters. J. Biol. Chem. 1996; (271) 30114-30120.

Diehl JA, Hannink M: Identification of a C/EBP-Rel complex in avian lymphoid cells. Mol Cell Biol. 1994; (14) 6635–6646.

Dlaska M and Weiss G. Central role of transcription factor NF-IL6 for cytokine and iron-mediated regulation of murine inducible nitric oxide synthase expression, J. Immunol. 1999 (162) 6171-7.

Esnault S, Shen ZJ, Whitesel E, Malter JS.The peptidyl-prolyl isomerase Pin1 regulates granulocyte-macrophage colony-stimulating factor mRNA stability in T lymphocytes.J Immunol. 2006; 177(10):6999-7006.

Esnault S, Braun RK, Shen ZJ, Xiang Z, Heninger E, Love RB, Sandor M, Malter JS.Pin1 modulates the type 1 immune response. PLoS ONE. 2007; 2(2):e226.

Farrow B, Evers BM. Inflammation and the development of pancreatic cancer. Surg. Oncol. 2002; (10) 153-169.

Feldmann M, Brennan FM, and Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol. 1996; (14) 397–440.

Feldmann M, Brennan FM, Chantry D, Haworth C, TurnerM, Abney E, Buchan G, Barrett K, Barkley D, Chu A, Field M, & Maini RN. Cytokine production in the rheumatoid joint: implications for treatment. Ann. Rheum. Dis. 1990; (49) 480 ± 486.

Firestein GS. Etiology and pathogenesis of rheumatoid arthritis, Kelley's Textbook of Rheumatology (7th ed.), W.B. Saunders, Philadelphia, PA. 2005; 996–1042.

Forstermann U, Kleinert H. Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn Schmiedebergs. Arch. Pharmacol. 1995 ;(352) 351-364.

Gilmore TD. The Rel/NF-κB signal transduction pathway: introduction. Oncogene. 1999; (18) 6842-6844.

Gius D, Botero A, Shah S, Curry HA. Intracellular oxidation/reduction status in the regulation of transcription factors NF- κB and AP-1. Toxicol. Lett. 1999; (106) 93-106.

Goetzl EJ, An S, Smith WL, Specificity of expression and effects of eicosanoid mediators in normal physiology and human diseases FASEB J. 1995; (9) 1051–1058.

Gohar AA, Lahloub ME, Niwa M. Antibacterial polyphenol from Erodium glaucophyllum. Zeitsch. Naturforsch. 2003; (58) 670-674.

Gorgoni B, Caivano M, Arizmendi C, Poli V. The transcription factor C/EBPβ is essential for inducible expression of the cox-2 gene in macrophages but not in fibroblasts. J. Biol. Chem. 2001; (276) 40769-40777.

Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal. 2001; (13) 85-94.

Han S, Sidell N, Roser-Page S, Roman J. Fibronectin stimulates human lung carcinoma cell growth by inducing cyclooxygenase-2 (COX-2) expression. Int J Cancer. 2004; 111(3):322-31.

Hayden MS, Ghosh S. Signaling to NF-κB Genes Dev. 2004; (18) 2195–2224.

Hiramatsu N, Xiufen W, Takechi R, Itoh Y, Mamo J. Antimutagenicity of Japanese traditional herbs, gennoshoko, yomogi, senburi and iwa-tobacco Pal S. Biofactors 2004; (22) 123-125.

Hla T, Neilson K, Human cyclooxygenase-2 cDNA, Proc. Natl.Acad. Sci. USA. 1992; (89) 7384–7388.

Holmdahl RAssociation of MHC and rheumatoid arthritis. Why is rheumatoid arthritis associated with the MHC genetic region? An introduction.Arthritis Res. 2000; (3):203-4. Review.

Holmdahl RM, Andersson TJ, Goldschmidt K, Gustafsson L, Jansson Land Mo JA. Type II collagen autoimmunity in animals and provocations leading to arthritis, Immunol. Rev. 1990 ;(118) 193.

Huang WC, Chen JJ, Chen CC. c-Src-dependent tyrosine phosphorylation of IKKβ is involved in tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 expression. J. Biol. Chem. 2003; (278) 9944-9952.

Huguet AI, Manez S, Alcaraz MJ. Superoxide scavenging properties of flavonoids in a non-enzymic system. Zeitschrift fur Naturforschung. 1990; (45) 19-24.

Ialenti A, Moncada S, & Di Rosa M. Modulation of adjuvant arthritis by endogenous nitric oxide. Br. J. Pharmacol. 1993; (110) 701 ± 706.

Jones PL, Ping D, Boss JM. Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-β and NF- κB. Mol Cell Biol. 1997; (17) 6970–6981.

Kamil M, Ilyas M, Rahman W, Hasaka N, Okigawa M, Kawano N. Taiwaniaflavone and its derivatives: a new series of biflavones from *Taiwania cryptomerioides* Hayata. J. Chem. Soc. Perkin. Trans. 1981 (1) 553-559.

Kang KW, Choi SY, Cho MK, Lee CH, Kim SG. Thrombin Induces Nitric-oxide Synthase via Gα12/13-coupled Protein Kinase C-dependent I-κB Phosphorylation and JNK-mediated I- κB Degradation. J. Biol. Chem. 2003; (278) 17368-17378.

Kang RY, Freire-Moar J, Sigal E, Chu CQ. Expression of cyclooxygenase-2 in human and an animal model of rheumatoid arthritis. Br J Rheumatol. 1996; 35:711–8.

Kanno S, Shouji A, Tomizawa A, Hiura T, Osanai Y, Ujibe M, Obara Y, Nakahata N, Ishikawa M. Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. Life Sci. 2006; (78) 673-681.

Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF- κB activity. Ann. Rev. Immunol. 2000; (18) 621-663.

Kauss T, Moynet D, Rambert J, Al-Kharrat A, Brajot S, Thiolat D,

Ennemany R, Fawaz F, Mossalayi MD. Rutoside decreases human macrophage-derived inflammatory mediators and improves clinical signs in adjuvant-induced arthritis. Arthritis Res Ther. 2008; 10(1):R19.

Ki SH, Choi MJ, Lee CH, Kim SG.Galpha12 specifically regulates COX-2 induction by sphingosine 1-phosphate. Role for JNK-dependent ubiquitination and degradation of I-κBα. J Biol Chem. 2007; 282(3):1938-47.

Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory Plant Flavonoids and Cellular Action Mechanisms. J. Pharmacol. Sci. 2004; (96) 229-245.

Kim Y, Fischer SM. Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells. Regulatory role of CCAAT/enhancerbinding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. J. Biol. Chem. 1998; (273) 27686–27694.

Kinugawa K, Shimizu T, Yao A, Kohmoto O, Serizawa T, Takahashi T. Transcriptional regulation of inducible nitric oxide synthase in cultured neonatal rat cardiac myocytes. Circ Res. 1997; 81(6):911-21.

Koon HW, Zhao D, Zhan Y, Rhee SH, Moyer MP, Pothoulakis C. Substance P stimulates cyclooxygenase-2 and prostaglandin E2 expression through JAK-STAT activation in human colonic epithelial cells. J Immunol. 2006; 176(8):5050-9.

Kosaka T, Miyata A, Ihara H, Hara S, Sugimoto T, Takeda O, Takahashi E, Tanabe T. Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2, Eur. J. Biochem. 1994; (221) 889–897.

Kratsovnik E, Bromberg Y, Sperling O, Zoref-Shani E. Oxidative stress activates transcription factor NF-kB-mediated protective signaling in primary rat neuronal cultures. J. Mol. Neurosci. 2005; (26) 27-32.

Kravchenko VV, Mathison JC, Schwamborn K. IKKi/IKKepsilon plays a key role in integrating signals induced by pro-inflammatory stimuli. J Biol Chem. 2003; (278)26612–26619.

Kristof AS, Goldberg P, Laubach V, Hussain SN. Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. Am. J. Resp. Crit. Care Med., 1998; (158)1883-1889.

Kubes P, McCaVerty DM. Nitric oxide and intestinal inflammation, Am. J. Med. 2000; (109) 150–158.

Kuo YC, Sun CM, Tsai WJ, Ou JC, Chen WP, Lin CY. Chinese herbs as modulators of human mesangial cell proliferation: preliminary studies. The J. Lab. Clin. Med. 1998; (132) 76-85.

Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol. 2001; (2) 149-156.

Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-α and COX-2 expression by sauchinone effects on I-κBα phosphorylation, C/EBP and AP-1 activation, Br. J. Pharmacol. 2003; (13) 11-20.

Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF- α and COX-2 expression by sauchinone effects on I-kappaBalpha phosphorylation, C/EBP and AP-1 activation. Br. J. Pharmacol. 2003; (139) 11-20.

Lee IS, Nishikawa A, Furukawa F, Kasahara K, Kim SU. Effects of Selaginella tamariscina on in vitro tumor cell growth, p53 expression, G1 arrest and in vivo gastric cell proliferation. Cancer Lett. 1999; (144) 93-99.

Lee JC, Kundu JK, Hwang DM, Na HK, Surh YJ. Humulone inhibits

phorbol ester-induced COX-2 expression in mouse skin by blocking activation of NF-kB and AP-1: IkB kinase and c-Jun-N-terminal kinase as respective potential upstream targets. Carcinogenesis. 2007; 28(7):1491-8.

Lee SJ, Hou J, Benveniste EN. Transcriptional regulation of intercellular adhesion molecule-1 in astrocytes involves NF-kB and C/EBP isoforms. J Neuroimmunol. 1998; (92)196–207.

Leng J, Yao H, Shen J, Wang K, Zhuo G, Wang Z. Co-expression of IL-18 binding protein and IL-4 regulates Th1/Th2 cytokine response in murine collagen-induced arthritis. Acta Biochim Biophys Sin (Shanghai). 2008; 40(2):116-24.

Liu QH, Jeong JE, Choi EJ, Moon YH, Woo ER. A new furofuran lignan from Geranium thunbergii Sieb. et Zucc. Arch. Pharm. Res.2006; (29) 1109-1113.

Liu T, Huang Y, Likhotvorik RI, Keshvara L, Hoyt DG. Protein Never in Mitosis Gene A Interacting-1 (PIN1) regulates degradation of inducible nitric oxide synthase in endothelial cells. Am J Physiol Cell Physiol. 2008; 295(3):C819-27.

Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature. 1996; (380) 544-547.

Lu KP. Pinning down cell signaling, cancer and Alzheimer's disease. Trends Biochem. Sci. 2004; (29) 200-209.

Lufei C, Koh TH, Uchida T, Cao X. Pin1 is required for the Ser727 phosphorylation-dependent Stat3 activity.Oncogene. 2007; 26(55):7656-64.

Lynch PM. COX-2 inhibition in clinical cancer prevention. Oncol. 2001; (15) 21-26.

Majithia V, Geraci SA. "Rheumatoid arthritis: diagnosis and management". Am. J. Med. 2007; 120 (11): 936–9.

Markham KR, Sheppard C, Geiger H. 13C NMR studies of some naturally occurring amentoflavone and hinokiflavone biflavonoids. Phytochem. 1987; (26) 3335-3337.

Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. Semin. Arthritis Rheum. 2003; (33) 155-167.

Miao N, Tao H, Tong C, Xuan H, Zhamg G. The *Selaginella tamariscina* Spring complex in the treatment of experimental diabetes and its effect on blood rheology. Zhongguo Zhong Yao Za Zhi 1996; (21) 493-495.

Monje P, Hernandez-Losa J, Lyons RJ, Castellone MD, Gutkind JS, Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. J. Biol. Chem. 2005; (280) 35081-35084.

Mori H, Xu Q, Sakamoto O, Uesugi Y, Koda A, Nishioka I. Mechanisms of antiumor activity of aqueous extracts from chinese herbs: Their imunopharmacological properties. Jpn. J. Pharmacol. 1989; (49) 423-431.

Moyers SB, Kumar NB. Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials. Nutr. Rev. 2004; (62) 204-211.

Muller JM, Ziegler-Heitbrock HW, Baeuerle PA. Nuclear factor kappa B, a mediator of lipopolysaccharide effects. Immunobiol. 1993; (187) 233-256.

Myers LK, Kang AH, Postlethwaite AE, et al. The genetic ablation of cyclooxygenase 2 prevents the development of autoimmune arthritis. Arthritis Rheum 2000; 43:2687-93.

Newton R, Kuitert LM, Bergmann M, Adcock IM, Barnes PJ. Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. Biochem.

Biophys. Res. Commun. 1997; (237) 28-32.

Ohshima H, Tatemichi M, Sawa T. Chemical basis of inflammation-induced carcinogenesis. Arch. Biochem. Biophys. 2003; (417) 3-11.

Okuda T, Yoshida T, Mori K. Consitutents of Geranium thunbergii Sieb. ET Zucc. II. Ellagitannins. (1) (Author's transl. J. Pharm. Soc. Jap. (Yakugaku Zasshi). 1975; (95)1462-1466.

Oyanagui Y. Nitric oxide and superoxide radical are involved in both initiation and development of adjuvant arthritis in rats. Life Sci. (1994) ;(54) PL285 ± PL289.

Pan MH, Lin-Shiau SY, Ho CT, Lin JH, Lin JK. Suppression of lipopolysaccharide-induced nuclear factor-kappaB activity by theaflavin-3,3'-digallate from black tea and other polyphenols through down-regulation of IkappaB kinase activity in macrophages, Biochem. Pharmacol. 2000; (59) 57-67.

Pang R, Lee TK, Poon RT, Fan ST, Wong KB, Kwong YL, Tse E. Pin1 interacts with a specific serine-proline motif of hepatitis B virus X-protein to enhance hepatocarcinogenesis. Gastroenterology. 2007 Mar; 132(3):1088-103.

Park SY, Hong SS, Han XH, Hwang JS, Lee D, Ro JS, Hwang BY.

Lignans from Arctium lappa and Their Inhibition of LPS-Induced Nitric Oxide Production. Chem. Pharm. Bull. 2007; (55) 150-152.

Pokharel YR, Liu QH, Aryal DK, Kim YG, Woo ER, Kang KW. 7,7'-Dihydroxy bursehernin inhibits the expression of inducible nitric oxide synthase through NF-.KAPPA.B DNA binding suppression. Nitric Oxide. 2007; (16)274-285.

Poli V. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J Biol Chem. 1998; (273)29279–29282.

Porsti I, Paakkari I, Nitric oxide-based possibilities for pharmacotherapy, Ann. Med. 1995; (27) 407–420.

Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP. Regulation of NF-kB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. Mol Cell. 2003 ;12(6):1413-26.

Salvemini D, Manning PZ, Zweifel BS, Seibert K, Connor J, Currie MG, Needleman P, & Masferrer JL. Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. J. Clin. Invest. (1995);(196) 307.

Salvemini D, Wang ZQ, Stern MK, Currie MG, & MiskoTP.

Peroxynitrite decomposition catalysts: therapeutics for peroxynitrite-mediated pathology. Proc. Natl. Acad. Sci. U.S.A. 1998; (95) 2659 ± 2663.

Sarkar FH, Li Y. Soy isoflavones and cancer prevention. Cancer Invest. 2003; (21) 744-757.

Schmedtje JF Jr, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. J. Biol. Chem. 1997; (272) 601-608.

Shen ZJ, Esnault S, Malter JS.The peptidyl-prolyl isomerase Pin1 regulates the stability of granulocyte-macrophage colony-stimulating factor mRNA in activated eosinophils.Nat Immunol. 2005; 6(12):1280-7.

Shinmei M, Masuda K, Kikuchi T, & Shimomura Y. Interleukin 1, tumor necrosis factor, and interleukin 6 as mediators of cartilage destruction. Semin. Arthritis Rheum. (1989); (18) 27 ± 32.

Siegle I, Klein T, Backman JT, Saal JG, Nusing RM, Fritz P. Expression of cyclooxygenase 1 and cyclooxygenase 2 in human synovial tissue: differential elevation of cyclooxygenase 2 in inflammatory joint diseases. Arthritis Rheum 1998; 41:122-9.

Southan GJ, Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms, Biochem. Pharmacol. 1996; (51) 383–394.

Stedman's Medical Dictionary, Twenty-fifth Edition, Williams & Wilkins, 1990.

Stein B, Cogswell PC, Baldwin AS Jr. Functional and physical associations between NF-kB and C/EBP family members: a Rel domain-bZIP interaction. Mol Cell Biol. 1993; (13)3964–3974.

Strunk V, Hahnenkamp K, Schneuing M, Fischer LG, Rich GF. Selective iNOS inhibition prevents hypotension in septic rats while preserving endothelium-dependent vasodilation. Anesth. Analg. 2001; (92) 681-687.

Stuart JM, Cremer MAI, & Kang AH. Type II collagen-induced arthritis in rats: passive transfer serum and evidence that IgG anticollagen antibodies can cause arthritis. J. Exp. Med. 1982a; (155) 1 ± 10.

Stuart JM, Townes AS, & Ang AH. The role of collagen autoimmunity in animals and human diseases. J. Invest. Dermatol. 1982b; (79) (Suppl): 1 ± 11.

Svensson L, Jirholt J, Holmdahl R, Jansson L. B cell-deficient mice do

not develop type II collagen-induced arthritis (CIA). Clin Exp Immunol 1998; 111:521–6.

Symmons DP. Epidemiology of rheumatoid arthritis: determinants of onset, persistence and outcome. Best Pract Res Clin Rheumatol. 2002; (5):707-22. Review

Szabo C. Role of nitric oxide in endotoxic shock. An overview of recent advances, Ann. N. Y. Acad. Sci. 1998; (851) 422-425.

Tak PP, and Firestein GS. NF-κB: a key role in inflammatory diseases, J Clin Invest. 2001; (107), 7-11.

Tamura M, Sebastian S, Yang S, Gurates B, Fang Z, Okamura K, Bulun SE. Induction of cyclooxygenase-2 in human endometrial stromal cells by malignant endometrial epithelial cells: evidence for the involvement of extracellularly regulated kinases and CCAAT/ enhancer binding proteins, J. Mol. Endocrinol. 2003; (31) 95–104.

Tan PH, Sagoo P, Chan C, Yates JB, Campbell J, Beutelspacher SC, Foxwell BM, Lombardi G, George AJ. Inhibition of NF- κB and Oxidative Pathways in Human Dendritic Cells by Antioxidative Vitamins Generates Regulatory T Cells. J. Immunol. 2005; (174) 7633-7644.

Tang Q, Chen W, Gonzales MS, Finch J, Inoue H, Bowden GT. Role of cyclic AMP responsive element in the UVB induction of cyclooxygenase-2 transcription in human keratinocytes. Oncogene. 2001; (20) 5164-5172.

Thomas B, Berenbaum F, Humbert L, Bian H, Bereziat G, Crofford L, Olivier JL. Critical role of C/EBP-beta and C/EBP-delta factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1 in articular chondrocytes. Eur. J. Biochem. 2000; (267) 6798-6809.

Trentham DE. Collagen arthritis as a relevant model for rheumatoid arthritis: evidence pro and con. Arthritis Rheum. 1982; (25) 911 ± 916.

Trushin SA, Pennington KN, Carmona EM, Asin S, Savoy DN Billadeau DD, Paya CV. Protein kinase Calpha (PKCalpha) acts upstream of PKCtheta to activate IkB kinase and NF-kB in T lymphocytes.Mol. Cell. Biol. 2003; (23) 7068-7081.

Tsuji S, Tsujii M, Kawano S, Hori M, Cyclooxygenase-2 upregulation as a perigenetic change in carcinogenesis, J. Exp. Clin. Cancer Res. 2001; (20) 117–129.

Ushio Y, Okuda T, Abe H. Effects of geraniin on morphology and

function of macrophages. Int. Arch. Allerg. Appl. Immunol. 1991; (196) 224-230.

Van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. J Exp Med. 1995; 181:775–9.

Van Rees BP, Saukkonen K, Ristimaki A, Polkowski W, Tytgat GN, Drillenburg P, Offerhaus GJ. Cyclooxygenase-2 expression during carcinogenesis in the human stomach. J. Pathol. 2002; (196) 171-179.

Wang T, Zhang X, Li JJ. The role of NF-kappaB in the regulation of cell stress responses. Int. Immunopharmacol. 2002; (2) 1509-1520.

Wardlaw SA, Zhang N, Belinsky SA. Transcriptional regulation of basal cyclooxygenase-2 expression in murine lung tumor-derived cell lines by CCAAT/enhancer-binding protein and activating transcription factor/cAMP response element-binding protein, Mol. Pharmacol. 2002; (62) 326–333.

Watanabe K, Kawamori T, Nakatsugi S, Wakabayashi K. COX-2 and iNOS, good targets for chemoprevention of colon cancer. Biofactors. 2000 (12) 129-133.

Weixing C, Qingbo T, Melissa SG and G Tim B. Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. 2001; (20) 29, 3921-3926

Westacott CI, Whicher JI, Barnes IC, Thompson D, Swan AJ, & Dieppe PA. Synovial fluid concentration of ®ve di€erent cytokines in rheumatic diseases. Ann. Rheum. Dis. 1990; 49ii, 678 ± 681.

Weiwad M, Küllertz G, Schutkowski M, Fischer G.Evidence that the substrate backbone conformation is critical to phosphorylation by p42 MAP kinase.FEBS Lett. 2000; 478(1-2):39-42.

Williams & Wilkins, 1990 Stedman's Medical Dictionary, Twenty-fifth Edition,

Woo ER, Lee JY, Cho IJ, Kim SG, Kang KW. Amentoflavone inhibits the induction of nitric oxide synthase by inhibiting NF-kappaB activation in macrophages. Pharmacol. Res. 2005; (51) 539-546.

Wu D, Marko M, Claycombe K, Paulson KE, Meydani SN. Ceramide-induced and age-associated increase in macrophage COX-2 expression is mediated through up-regulation of NF-kappa B activity. J. Biol. Chem. 2003; (278) 10983–10992.

Wulf GM, Ryo A, Wulf GG, Lee SW, Niu T, Petkova V, Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. EMBO J. 2001; (20) 3459-3472.

Xiao C, and Ghosh S. NF-κB, an evolutionarily conserved mediator of immune and inflammatory responses, Adv Exp Med Biol. 2005; (560), 41-55.

Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem. 1994; 269(7):4705-8.

Xie QW, Whisnant R, Nathan C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon-γ and bacterial lipopolysaccharide. J. Exp. Med. 1993; (177) 779-784.

Xiufen W, Hiramatsu N, Matsubara M. The antioxidative activity of traditional Japanese herbs. Biofactors. 2004; (21) 281-284.

Yang F, Oz HS, Barve S, Villiers WJ, McClain CJ, Varilek GW. The green tea polyphenol (-)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6. Mol. Pharmacol. 2001;

(60) 528-533.

Yang JW, Pokharel YR, Kim MR, Woo ER, Choi HK, Kang KW. Inhibition of inducible nitric oxide synthase by sumaflavone isolated from Selaginella tamariscina. J. Ethnopharmacol. 2006; (105) 107-113.

Yannis Alamanos, Paraskevi V. Voulgari D, and Alexandros A. Drosos, FACR. Incidence and Prevalence of Rheumatoid Arthritis, Based on the 1987 American College of Rheumatology Criteria: A Systematic Review.

Yoo TL, Kim SY, Stuart LM, Floyd RA, Olson GA, Cremer MA, Kang, AM. Induction of arthritis in monkeys by immunisation with type II collagen. J. Exp. Med. 1988; (168) 777 ± 782.

Yu X, Kennedy RH and Liu SJ. JAK2/STAT3, not ERK1/2, mediates interleukin-6-induced activation of inducible nitric-oxide synthase and decrease in contractility of adult ventricular myocytes. J Biol Chem. 2003; 278:16304.

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell. 1997; (91) 243-252.

Zha S, Yegnasubramanian V, Nelson WG, Isaacs WB, De Marzo AM. Cyclooxygenases in cancer: progress and perspective. Cancer Lett. 2004 (215) 1-20.

Zhou XZ, Kops O, Werner A, Lu PJ, Shen M, Stoller G, Küllertz G, Stark M, Fischer G, Lu KPPin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. Mol Cell. 2000; 6(4):873-83.

국문 초록

염증매개물의 유도과정에서 Pin1의 역할 및 천연물의 항염증효과연구

유바 라쥐 포카렐

지도 교수: 강 건 욱

조선대학교 대학원 약학과

류마티스 관절염은 관절의 만성염증에 이어 연골과 뼈의 파괴가 일어나는 자가면역질환이다. Prostaglandins과 proinflammatory cytokines과 같은 면역매개물질이 류마티스 관절염에 관여한다고 보여지고 있다. Peptidyl prolyl isomerase인 Pin1은 암이나 신경퇴행성질환과 같은 몇몇 질환에서 중요한 생리학적 작용을 한다. 우리는 Type Ⅱ collagen-induced RA mice에서 Pin1과 cyclooxygenase-2 (COX-2)가 높게 발현되어 있는 것을 발견했다. GFP-overexpressed cells에 비해 Pin1-overexpressed HTB-94 cells and 초기 배양된 인간 연골조직에서 proinflammatory proteins (COX-2, inducible

nitric oxide synthase, tumor necrosis factor-α and interleukin-1β)이 매우 증가되어 있었다. Site-specific mutation analyses에 의해 Pin1에 의한 COX-2 유전자의 전사적 활성이 nuclear factor-κB (NF-κB), cyclic AMP response element binding protein (CREB) 그리고 CCAAT-enhancer binding protein 에 의해서도 조절된다는 것을 알 수 있었다. Gel shift, reporter gene 과 Western blot analyses를 이용하여 Pin1-overexpressed chondrocyte cell C/EBP line 에서 NF-κB, CREB이 동등하게 활성화 된다는 것을 확인하였다. Pin1의 화학적 억제제인 juglone을 투여한 RA mice의 발목조직에서 RA progress 그리고 COX-2 발현이 매우 감소되었다. 게다가 류마티스 환자의 초기 배양된 인간연골조직에서 COX-2의 발현이 juglone의 농도에 의존적으로 감소 되었다. 이러한 결과들로 류마티스 관절염의 진행 중 Pin1의 합성이 NF-κB, CREB, C/EBP 그리고 AP-1에 의해 proinflammatory protein에 자극이

되고, Pin1이 류마티스 관절염의 치료의 중요한 표적이 된다고 생각하게 되었다.

Inducible nitric oxide synthase (iNOS) 그리고 cyclooxygenase-2 (COX-2)의 유도에 의한 비정상적 NO 와 prostaglandin 의 생산이 만성염증의 발생에 관여한다. *Selaginella tamariscina* 는 동양의학에서 염증의 치료효과를 위해 사용 되어져 왔다. 우리가 *S. tamariscina* 에서 추출해낸 Taiwaniaflavone 과 2', 8″-biapigenin 이 lipopolysaccharide (LPS)로 자극시킨 RAW264.7 대식세포에서 iNOS와 COX-2의 합성에 영향이 있는지 실험 하였다. 우리는 Taiwaniaflavone이 p65의 핵으로의 이동을 억제하여 nuclear factor-κB 를 불활성화 시켜 iNOS와 COX-2 유전자의 전사활성을 억제한다는 것을 발견하였다. NF-κB의 활성이 I-κB의 파괴와 그에 따른 인산화에 의해서 일어난다는 것은 잘 알려진 사실이다. 그리고 이번 연구에서 Taiwaniaflavone이 I-κB의 파괴와 인산화를 억제한다

는 것을 실험하였다. 우리의 실험의 결과는 Taiwaniaflavone 와 2', 8"-biapigenin이 염증성질환 진행의 예방에 관여한다는 것을 뜻한다.

우리는 최근에 *Geranium thunbergii* 에서 새로운 리그난 물질인 4-hydroxykobusin 을 분리했다.(Liu et al., 2006). 여기서 우리는 이것이 RAW264.7 세포에서 *inducible nitric oxide synthase* (*iNOS*) 유전자의 발현에 영향을 준다는 것을 연구하였다. 4-hydroxykobusin은 농도의존적으로 LPS에 의한 inducible nitric oxide synthase (iNOS)의 발현을 차단하여 NO의 생성을 억제하였다. iNOS억제의 기전을 명확하게 하기 위해 –1.59 kb flanking region 을 이용한 luciferase reporter의 활성을 통하여 4-hydroxykobusin의 iNOS유전자의 전사활성을 실험 하였다. 이 Lignan은 reporter gene의 활성을 억제 하였으며 LPS에 의한 NF-κB 와 AP-1 reporter 활성의 증가 또한 현저하게 차단되었다. 이러한 발견은 LPS에 의한 NO 합성의 억제

가 NF-κB 와 AP-1의 활성의 억제에 의한 것임을 의미한다.

My Published Papers

1. **Pokharel YR,** Yoon SY, Kim SK, Li JD, Kang KW. Inhibition of acrolein-stimulated MUC5AC production by fucoidan in human bronchial epithelial cells.Pharmazie. 2008 Oct; 63(10):757-9.
2. **Pokharel YR**, Liu Oh, Oh JW, Woo ER, Kang Keon Wook. 4-Hydroxykobusin Inhibits the Induction of Nitric Oxide Synthase by Inhibiting NF-kappaB and AP-1 Activation.Biol Pharm Bull. 2007 Jun; 30(6):1097-101.
3. **Pokharel YR** and Kang KW. Ginsenoside Rd enhances glutathione levels via NF-kappaB-dependent gamma-glutamylcysteine ligase induction. BK21 Project Team, College of Pharmacy, Chosun University. Pharmazie. 2007 Dec; 62(12):933-6.
4. **Pokharel YR**, Lim SC, Kim SC, Heo TH, Choi HK and Kang KW. Sopungyangjae-Tang Inhibits Development of Dermatitis in Nc/Nga Mice. Evidence -based Complementary & Alternative Medicine. 2008 Jun; 5(2):173-180.
5. **Pokharel YR**, Liu QH, Aryal DK, Kim YG, Woo ER, Kang KW.

7, 7’-Dihydroxy bursehernin inhibits the expression of inducible nitric oxide synthase through NF-kappaB DNA binding suppression. Nitric Oxide. 2007 Mar; 16(2):274-85. Epub 2006 Oct 21.

6. **Pokharel YR**, Jeong JE, Oh SJ, Kim SK, Woo ER, Kang KW. Screening of potential chemopreventive compounds from Poncirus trifoliataRaf. Pharmazie. 2006 Sep; 61(9):796-8.

7. **Pokharel YR**, Han EH, Kim JY, Oh SJ, Kim SK, Woo ER, Jeong HG, Kang KW. Potent protective effect of isoimperatorin against aflatoxin B1-inducible cytotoxicity in H4IIE cells: bifunctional effects on glutathione S-transferase and CYP1A.Carcinogenesis. 2006 Dec; 27(12):2483-90. Epub 2006 Jul 8.

8. **Pokharel YR**, Yang JW, Kim JY, Oh HW, Jeong HG, Woo ER, Kang KW. Potent inhibition of the inductions of inducible nitric oxide synthase and cyclooxygenase-2 by taiwaniaflavone. Nitric Oxide. 2006 Nov; 15(3):217-25. Epub 2006 Feb 20(Top 25 most downloaded article in whole year 2006).

9. **Pokharel YR**, Lim SC, Kim SC, Choi HK, Kang KW. Inhibition of Dermatitis Development by Sopungsan in Nc/Nga Mice.

Toxicological Research Vol.24 No.1, 2008. 3, pp. 17 ~ 22

10. Kim ND, **Pokharel YR,** Kang KW. Ginsenoside Rd enhances glutathione levels in H4IIE cells via NF-kappaB-dependent gamma-glutamylcysteine ligase induction. Pharmazie. 2007 Dec; 62(12):933-6.
11. Kim SK, **Pokharel YR**, Kim O, Woo ER and KangKW. Inhibition of the Induction of Nitric Oxide Synthase by Kobusin. College of Pharmacy and Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon 305-764,College of Pharmacy, Chosun University, Gwangju 501-759, Korea. J. Toxicol. Pub. Health.Vol. 23, No. 2, pp. 123-126 (2007).
12. Jeong HG, **Pokharel YR.** Inhibition of cycloxygenase-2 by ginsenoside Rd via activation of CCAAT-enhancer binding proteins and cyclic AMP response binding protein. BK21 Project Team, College of Pharmacy, Chosun University, Seosuk-dong, Dong-gu, Gwangju 501-759, South Korea.(Online In BBRC). Biochem Biophys Res Commun. 2007 Jul 20; 359(1):51-6.
13. Woo ER, **Pokharel YR**, Yang JW, Lee SY, Kang KW.

Inhibition of nuclear factor-kappaB activation by 2', 8''-biapigenin.Biol Pharm Bull. 2006 May; 29(5):976-80.

14. Kang KW, Wagley Y, Kim HW, **Pokharel YR**, Chung YY, Chang IY, Kim JJ, Moon JS, Kim YK, Nah SY, Kang HS, Oh JW. Novel role of IL-6/SIL-6R signaling in the expression of inducible nitric oxide synthase (iNOS) in murine B16, metastatic melanoma clone F10.9, cells.Free Radic Biol Med. 2007 Jan 15; 42(2):215-27. 2006 Oct 12.
15. Yang JW, **Pokharel YR**, Kim MR, Woo ER, Choi HK, Kang KW. Inhibition of inducible nitric oxide synthase by sumaflavone isolated from Selaginella tamariscina.J Ethnopharmacol. 2006 Apr 21; 105(1-2):107-13. Epub 2005 Nov.

Papers to be Published

1. **Yuba Raj Pokharel** and Keon Wook Kang.Reversal of Ginsenosides on Multidrug resistance in Drug-resistant Human Breast cancer Cell line MCF7/ADR.
2. Hoo-Kyun Choi, **Yuba Raj Pokharel**, Hyo-Kyung Han, Chang Seon Ryu, Sang Kyum Kim, Sung Chul Lim, Mi Kyong Kwak and Keon Wook Kang**.** Inhibition of liver fibrosis by

solubilized coenzyme Q10: Possible role of Nrf2 activation in the inhibition of transforming growth factor-β1 expression**.**

3. **Yuba Raj Pokharel**, Sung Chul Lim, Yong Pil Hwang, Eun Hee Han, Kwang Yeol Lee, Hye Gwang Jeong, and Keon Wook Kang. Novel role of Pin1 induction in type II-collagen-mediated rheumatoid arthritis.

Conference and seminars:

1. **Pokharel YR** and Kang KW, Ginsenoside Rd enhances glutathione levels via NF-kappaB-dependent gamma-glutamylcysteine ligase induction. BK21 Project Team, College of Pharmacy, Chosun University. American Association of Pharmaceutical Scientists, 2007 AAPS Annual Meeting and Exposition, November 11-15, San-Diego Convention center San-Diego, USA.
2. **Pokharel YR** and KangKW.Ginsenoside Rd enhance glutathione levels via NF-kappaB-dependent gamma-glutamylcysteine ligase induction. BK21 Project Team, College of Pharmacy, Chosun University. 19th Federation of Asian and Oceanian Biochemist and Molecular biologist (FAOBMB), Seoul conference, May 27-30, 2007, COEX center, Seoul, Republic of Korea.
3. **Pokharel YR**, Yang JW, Kim JY, Oh HW, Jeong HG, Woo ER, Kang KW. Potent inhibition of the inductions of inducible nitric oxide synthase and cyclooxygenase-2 by taiwaniaflavone. College of Pharmacy Chosun University. 54th Proceeding of the convention of the pharmaceutical society of Korea. April16-17, 2006, Bexco, Busan, Korea.
4. **Pokharel YR**, Kang Keon Wook. Sopungyangjae-Tang inhibits

development of Dermititis in Nc/Nga mice. College of Pharmacy Chosun University.54thProceeding of the convention of the pharmaceutical society of Korea. April16-17, 2006, Bexco, Busan, Korea. Woo ER, **Pokharel YR**, Yang JW and Kang KW.Inhibition of nuclear factor- kB activation by 2'8''-biapigenin. College of Pharmacy Chosun University. 54th Proceeding of the convention of the pharmaceutical society of Korea. April16-17, 2006, Bexco, Busan, Korea.

5. **Pokharel YR**, Liu Quing-He, Woo EUn-Rahn, Kang Keon Wook.7, 7'-Dihydroxy bursehernin inhibits the expression of inducible nitric oxide synthase through NF-kappaB DNA binding suppression. 55th Proceeding of the convention of the pharmaceutical society of Korea. November 6-7, 2006, Seoul Education center, Seoul, Korea.
6. Kang KW, Wagley Y, Kim HW, **Pokharel YR**, Chung YY, Chang IY, Kim JJ, Moon JS, Kim YK, Nah SY, Kang HS, Oh JW.Novel role of IL-6/SIL-6R signaling in the expression of inducible nitric oxide synthase (iNOS) in murine B16, metastatic melanoma clone F10.9, cells. College of Pharmacy Chosun University. 55th Proceeding of the convention of the pharmaceutical society of Korea. November 6-7, 2006, Seoul Education center, Seoul, Korea.
7. Kang Keon Wook, **Yuba Raj Pokharel** and Woo Eun-Rhan. 4-Hydroxykobusin inhibits the induction of nitric oxide synthase by inhibiting NF-KB and NF-KB activation College of Pharmacy Chosun University. Proceeding of the convention of the pharmaceutical society of Korea. May11-12, 200, Kimdaejung Convention Center, Gwangju, Korea.
8. **Yuba Raj Pokharel** and Keon Wook Kang. Induction of cycloxygenase-2 by ginsenoside Rd via activation of CCAAT-enhancer binding proteins and cyclic AMP response binding

protein. College of Pharmacy Chosun University. Proceeding of the convention of the pharmaceutical society of Korea. May11-12, 200, Kimdaejung Convention Center, Gwangju, Korea.

9. **Yuba Raj Pokharel** and Keon Wook Kang.Inhibition of dermatitis development by Sopungsan in Nc/Nga mice. College of Pharmacy Chosun University. Proceeding of the convention of the pharmaceutical society of Korea. May11-12, 200, Kimdaejung Convention Center, Gwangju, Korea.

Acknowlegement

I would like to take this opportunity to extend my sincere gratitude to my advisor, Professor Dr. Keon Wook Kang for his guidance, inspiration and encouragement throughout my PhD study. Not only did I benefit from his profound experience, enormous enthusiasm, and keen insight in the subject matter, but I also profited from his wonderful personality. In my own word the best supervisor I could ever have wished for. I would like to sincerely thank to Professor Choi Hoo Kyun for his support and encouragement in my study. I want to acknowledge Prof. Jeong Hye Gwang, Dr. Choi Hong Seok, Dr. Oh Won Keun and Dr Lee Kwang Yeol for their time and effort while serving on my committee. I would also like to sincerely thank to all professors of college of Pharmacy who always gave me moral support to complete my PhD. There are many lab mates who worked together over 4 years of my research and spend their time and efforts with me. Special thanks goes to Kim Sang Eun, Kim Mira, Cho Kyoung Bin, Kim Jung Woo, Kim Ok, Ngyuyen ThiThuy Phuong as well as former fellows Yang Jin Won, Han Chang Yeob, Roh Sang Hee and Lee Jeong Yong for pleasant working atmosphere and many enjoyable moments and events. I would like to give special thanks for my friend Krishna Nath who introduced me in Korea for study. It would not be fair if I don't thank for the faculty, staff and students of college of Pharmacy and international affairs team of Chosun University for their help and support. I am really thankful to Kim Young Seoub who supported me as brother to adjust in Korea. I would like to thanks to all my Korean and Nepali friends who have supported me during my study in Korea.

I am extremely grateful to my parents for teaching me about

life, for making me to be a better person, and loving me unconditionally no matter what. My younger siblings are always a source of love and help, and I am very proud of them. I am also like to thank my uncles for their encouragements and enthusiasm. I would like to acknowledge the help and support of my parents- in – laws, who have treated me as their own son. Finally, I have to thank my wife, Sindhu. Without her steadfast love, support and help, it is impossible to complete my thesis successfully. I have to thank to my lovely daughter Shreeya and lovely son Yusin. They are the source of inspiration to help me and overcome any challenge that I have confronted.

저작물 이용 허락서

학 과	약학과	학 번	20057827	과 정	석사, **박사**
성 명	한글: 유바라쥐 포카렐 한문: 영문: Yuba Raj Pokharel				
주 소	Okharkot-8, Pyuthan, Nepal				
연락처	E-MAIL : metropolitan10@yahoo.com				
논문제목	**한글**: 염증매개물의 유도과정에서 Pin1 의 역할 및 천연물의 항염증효과연구. 영어: Role of Pin1 in the induction of proinflammatorymediators and studies on the anti-inflammatory effects of phytochemicals.				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부: 동의(○) 반대()

2009 년 2 월 25 일

저작자: 유바라쥐 포카렐

(서명 또는 인)

조선대학교 총장 귀하