African Journal of Biotechnology Vol. 11(7), pp. 1690-1699, 24 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2632
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Anti-Inflammatory molecular mechanisms of Bo-Ye-Niu-Pi-Xiao (*Cynanchum taiwanianum* Yamazaki)

Houi Lee[1], Tzuching Wang[2*], Tachen Lin[3], Jiahsin Guo[1], Chiching Yang[1] and Yingpei Shen[2,4]

[1]Department of Food Science and Technology, National Pingtung University of Science and Technology, Pingtung, 91201, Taiwan, ROC.
[2]Department of Food and Beverage Management, Tzuhui Institute of Technology, Pingtung 92641, Taiwan, ROC.
[3]Department of Tourism and Leisure Management, Fortune Institute of Technology, Kaohsiung City 83160, Taiwan, ROC.
[4]Ph.D Program in Management of Dayeh University, Changhua 51591, Taiwan, ROC.

Accepted 9 November, 2011

Bo-Ye-Niu-Pi-Xiao, *Cynanchum taiwanianum* Yamazaki (family: Asclepiadaceae) is a well-known and popular herb; its rhizome has been used as a folk medicine in Taiwan. Many therapeutic effects of *C. taiwanianum* Yamaz. had been studied; however, there is still no anti-inflammatory effect and mechanism of *C. taiwanianum* Yamaz. reported. Besides, it is well-known that the phosphorylation of I kappa B-alpha (IκBα) to phospho-IκBα (p-IκBα) is a decisive step in the nuclear factor kappa B (NF-κB) activation pathway. In addition, the induction of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) by cytokines, such as interleukin-1β (IL-1β), is well known to be partly mediated by NF-κB. Hence, the objective of this study was to evaluate the activity and distinguish the mechanism of anti-inflammation of *C. taiwanianum* Yamaz. rhizome on rat kidney cell line NRK-52E induced by IL-1β. In the present study, we investigated the anti-inflammatory effect and potential mechanism of *C. taiwanianum* Yamaz. rhizome ethanolic extract (CTEE) in IL-1β-induced NRK-52E cells. Production of nitric oxide (NO) and prostaglandin E2 (PGE_2) by enzyme linked immunosorbent assay. The messenger ribonucleic acid (mRNA) and protein expression of iNOS and COX-2, phosphorylation of IκBα, and activation of NF-κB was executed by reverse transcription-polymerase chain reaction and Western blotting. Results show that the CTEE significantly ($P < 0.05$) inhibited NO and PGE_2 production and also significantly ($P < 0.05$) attenuated protein and mRNA expression of iNOS and COX-2 in IL-1β-induced NRK-52E cells without obvious cytotoxic effects. Furthermore, the CTEE suppressed the NF-κB nuclear translocation, in terms of inhibition of IκBα phosphorylation. The results provided evidence for its folkloric uses and suggest that the anti-inflammatory activities of CTEE may result from the inhibition of inflammatory mediators, such as NO and PGE_2 and an upstream suppression of NF-κB-dependent mechanism might be involved.

Key words: *Cynanchum taiwanianum* Yamazaki, anti-inflammatory, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS).

INTRODUCTION

Inflammation reaction is not only the response of living tissues to infection and injury but also relevant to diseases including atherosclerosis and cancer (Coussens and Werb, 2002). Several reports indicated that over-production of nitric oxide (NO) are cytotoxic and are in relation to cell injury or tissue damage in a number of diseases such as inflammation and carcinogenesis (Mordan et al., 1993). Therefore, to find a potent nitric oxide synthase (NOS), inhibitor might be an effective therapeutic strategy for inflammation-related diseases (Koo et al., 2001). In inflammatory tissues, prostaglandin E2 (PGE_2) is also one of the major mediators and is a well-known oncogenic signal (Narumiya et al., 1999).

*Corresponding author. E-mail: tcw0511@gmail.com. Tel: +886-8-8647367 x 333. Fax: +886-8-8647123.

In the inflammation process, two inducible enzymes, that is, the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), are accountable for the synthesis of NO and PGE_2 (Sripanidkulchai et al., 2009), respectively. Recently, the activities of these two enzymes could be inhibited by several plant extracts such as wild bitter gourd (Lii et al., 2009) and green tea (Nakagawa and Yokozawa, 2002). At inflamed sites, NO and PGE_2 have pleiotropic-effects and produced respectively by COX-2 and iNOS which are induced in response to different stimuli including cytokines (Smith et al., 2000). Therefore, the inhibitors of these two enzymes may also be considered as anti-inflammatory agents.

It is well-known that the phosphorylation of I kappa B-alpha (IκBα) result in the degradation of IκBα and consequent dissociation of nuclear factor-kappa B (NF-κB) from IκBα (Hayden and Ghosh, 2004). Subsequently, the free NF-κB translocates into the nucleus and then activates its target genes (Vinayagamoorthi et al., 2005), including iNOS and COX-2 (Stylianou et al., 1998) and then increasing the expression of inflammatory mediators (La et al., 2003). Hence, phosphorylation of IκBα to phospho-IκBα (p-IκBα) is a decisive step in the NF-κB activation pathway (Yin et al., 1998). In addition, the induction of COX-2 and iNO synthase by cytokines, such as interleukin-1β (IL-1β), is well known to be partly mediated by NF-κB (Newton et al., 1997).

Bo-Ye-Niu-Pi-Xiao, *Cynanchum taiwanianum* Yamazaki (family: Asclepiadaceae) is a well-known and popular herb, which its rhizome has been used as a folk medicine in Taiwan. Although many therapeutic effects of *C. taiwanianum* Yamaz. had been studied, such as antifebrile, diuretic, antitussive, expectorant, anodyne, and tonic activities in China (Chen et al., 1991) and hepatoprotection in Taiwan (Lin et al., 1995), there is still no anti-inflammatory effect and mechanism of *C. taiwanianum* Yamaz. reported. Recently, a number of researchers focused on the chemical constituents (Lin et al., 1998) and potential biologically active compounds of *C. Taiwanianum* Yamaz. (Chen et al., 1991); however, little is studied about its actual effects and mechanisms of the aforementioned pharmacological activities. The objective of this study is to evaluate the activity and distinguish the mechanism of anti-inflammation of *C. taiwanianum* Yamaz. rhizome ethanolic extract (CTEE) on rat kidney cell line NRK-52E induced by interleukin 1-β (IL-1β).

MATERIALS AND METHODS

Plant material

The *C. taiwanianum* rhizome was collected from Neimen region, Kaohsiung, in Southern Taiwan, in April 2008. The plant was identified by Dr. Yan of the National Museum of Natural Science, Taichung, Taiwan. The voucher specimen of *C. taiwanianum* was deposited in Department of Management and Utilization, Fengshan Tropical Horticultural Experiment Branch. The herbarium number is FTHES-08-01. The cleaned rhizome was subjected to slicing, hot air-drying (50°C) and then grinding into powder. The ethanolic extract of *C. taiwanianum* was prepared as follows. 100 g of the *C. taiwanianum* powder was soaked in 500 ml 95% ethanol at 60°C and shaken for 1 h. The *C. taiwanianum* ethanolic extract (CTEE) was concentrated using a vacuum concentrator and then subjected to freeze-drying. The percentage yield of the ethanolic extract was 2.2% (w/w). The CTEE was filtered through a 0.45 μm filter and stored at 4°C until use.

Chemicals and regents

Anti-serum iNOS, I-κB, p-IκB and NF-κB were purchased from Santa Cruz Biotechnology Inc. (CA, USA). Avidin-horseraddish perxidase (Av-HRP) was obtained from BD Pharmingen Inc. (California, USA). Bovine serum albumin, sodium bicarbonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) formazan, sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride, ethidium bromide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), potassium chloride, magnesium chloride, ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, NP-40 lysis buffer (NP-40), 3,3-diaminobenzidine (DAB), β-actin and glycerol were purchased from Sigma Chemical Co. (MO, USA). COX-2, PGE_2 and PGE_2-acetylcholineesterase conjugate were obtained from Cayman Co. (Ann Arbor, MI, USA). Isopropanol was obtained from Merck Chem. Inc. (Germany). Easy-BLUE™ RNA extraction kit was obtained from INtRON Biotechnology Inc. (Korea). Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), TRI_{ZOL}® reagent, penicillin-streptomycin and trypsin-EDTA were purchased from GIBCO-BRL (NY, USA). Interleukin-1β (IL-1β) was obtained from R&D Systems Inc. (MN, USA). Taq DNA polymerase, oligo-dT primer and dNTPs were obtained from Invitrogen Co. (NY, USA).

Cell culture

The normal rat kidney cell line NRK-52E (BCRC 60086) was purchased from Bioresource Collection and Research Center of Food Industry Research and Development Institute in Taiwan and maintained in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine, at 37°C in 5% CO_2 humidified air. Cells were preincubated with and without various concentration of CTEE (0.1, 0.5, 1.0 mg/ml) followed by IL-1β (5 ng/ml) 30 min later. Supernatants were collected after IL-1β treatment for 18 h for NO/PGE_2 determination, respectively.

Cell viability assay

Cell viability was evaluated by the mitochondrial-dependent MTT reduction method. The CTEE-treated cells were washed with phosphate buffer saline (PBS) twice and the medium was replaced with DMED without containing phenol red. MTT solution (0.5 mg/ml) was added to the cell cultures. After incubation for 3 h at 37°C, the medium was removed again and the formazan crystals in viable cells were dissolved with 1 ml of isopropanol. After 10 min shaking, the solution was centrifuged at 5000 × *g* for 5 min. The absorbance of the supernatant of each sample was determined at 570 nm. The optical density of formazan formed in control (untreated) cells was taken as 100% viability.

Stimulation experiment

Cells were harvested by gentle scraping, plated into 24-well plates

at a density of 8 × 10^4 cells per well, and allowed to adhere for 24 h at 37°C under 5% CO_2 atmosphere. For stimulation, the culture medium was replaced with fresh DMEM containing 10% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 1% L-glutamine in the presence or absence of 5 ng/ml IL-1β. To evaluate the effects of the extracts, cells were first incubated with the CTEE at the concentrations of 0.1, 0.5 or 1.0 mg/ml for 5 h, and then with or without 5 ng/ml IL-1β as aforementioned for 18 h. As reference controls, assays were also performed with medium containing PBS and IL-1β only.

Nitrite assay

As an indicator of NO production, we determined the nitrite (NO_2^-) concentration in the culture medium by the Griess reaction (Green et al., 1982). 100 µl of each culture supernatant, assayed in triplicate, was reacted with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid solution) at room temperature for 10 min under dark. Absorbance was measured at 540 nm against a calibration curve with sodium nitrite standards.

PGE_2 measurement

PGE_2 production was measured in culture medium in order to determine COX-2 activity. The culture medium of control and treated cells was collected, centrifuged, and stored at -80°C until tested. The level of PGE_2 released into culture medium was quantified by using a commercial competitive enzyme immunoassay kit (EIA, Cayman Chemical, Co., Ann Arbor, MI, U.S.A.) according to the manufacturer's instruction.

Western blotting

Whole cell extracts containing equal quantities of proteins (30 to 50 µg) were electrophoresed in 10% polyacrylamide gel. Subsequently, the separated proteins were transferred to polyvinylidene fluoride membrane using Semi-Dry Transfer Cell (TRANS-BLOT, BIO-RAD, U.S.A.). Briefly, the membrane was blocked for 30 min with blocking buffer (5% skim milk in 50 mM Tris-HCl, 200 mM NaCl, and 0.05% Tween 20, pH 7.5), and was incubated with appropriate dilutions of primary antibodies (against iNOS, COX-2, IκB, p- IκB and NF-κB) for overnight at 4°C. After washing twice with the aforementioned buffer, the membrane was further incubated for 2 h with 1:1000 dilution of biotin-conjugated goat anti-rabbit or anti-mouse antibody (Biotain Co., Dubulin, Ireland), and developed with Av-HRP and DAB solution. In the case of COX-2, an enhanced chemiluminescence (ECL) method was used as Western blot detection system (Amersham Biosciences, Inc., Piscataway, NJ, U.S.A.) according to the manufacturer's instruction.

Nuclear protein preparation

Cells were preincubated with each of the CTEE for 3 h before the addition of 5 ng/ml IL-1β for 90 min. Cells were then washed twice and scraped with cold PBS and centrifuged. The pellets were resuspended in the hypotonic extraction buffer containing 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 4 µg/ml leupeptin, 20 µg/ml aprotinin, and 0.5% NP-40 for 15 min on ice and were then centrifuged at 6000 × *g* for 15 min. Nuclear proteins were extracted by gently mixing with 50 µl hypertonic extraction buffer containing 10 mM HEPES, 0.4 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 4 µg/ml leupeptin, 20 µg/ml aprotinin, and 10% glycerol at 4°C for 30 min. The samples were then centrifuged at 10,000 × *g* for 15 min. The supernatant fluid containing the nuclear proteins was collected for nucleic protein assay.

RNA extraction and reverse transcription-polymerase Chain Reaction (RT-PCR)

Total cellular RNA was isolated using an easy-BLUE™ RNA extraction kit according to the manufacturer's instructions. Briefly, total cellular RNA was extracted with TRI_{ZOL}® reagent (Gibco Laboratories, Inc., Grand Island, NY, U.S.A.) according to the manufacturer's instructions. RNA concentrations were calculated from absorbance at 260 and 280 nm. Total RNA (2 mg) was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo-dT primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM dNTPs at 42°C for 1 h. The reaction was then stopped by incubating the solution at 70°C for 15 min, after which 3 ml of the cDNA mixture was used for enzymatic amplification. PCR was then performed using a reaction mixture comprised of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 m M each of primers specific for iNOS, COX-2, and β-actin. The amplification conditions were as follows: Denaturation at 94°C for 3 min for the first cycle and then 35 cycles of 94°C for 45 s, annealing of iNOS at 56°C for 45 s or annealing of COX-2 at 53°C for 45 s with a final extension at 72°C for 7 min. The PCR products were then electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. β-actin which served as an internal control. The primers used in this study were as follows: iNOS, 5′-AGCCCAACAATACAAATGACCCTA-3′ (sense) and 5′-TTCCTGTTGTTTCTATTTCCTTTGT-3′ (antisense); Cox-2, 5′-CACTCAGTTTGTTGAGTCATTC-3′ (sense) and 5′-GATTAGTACTGTAGGGTTAATG-3′ (antisense); β-actin, 5′-ATGAAGATCCTGACCGAGCGT-3′ (sense) and 5′-AACGCAGCTCAGTAACAGTCCG-3′ (antisense).

Statistical analysis

The control and treatment groups were compared by ANOVA. Duncan's *t*-test was used for evaluating statistical significance. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of CTEE on cell viability

To exclude possible cytotoxic effect of CTEE on NRK-52E cells in either the presence or the absence of IL-1β, the MTT assay was employed. Cell viabilities of NRK-52E cells were not significantly altered and the cell viabilities were above 95% up after 18 h incubation with various concentrations of CTEE (0.1, 0.5, and 1.0 mg/ml) in the presence or absence of IL-1β (data not shown).

Effect of CTEE on IL-1β-induced NO and PGE_2 production

To estimate the effects of CTEE on IL-1β-induced NO and PGE_2 production in NRK-52E cells, cells were treated

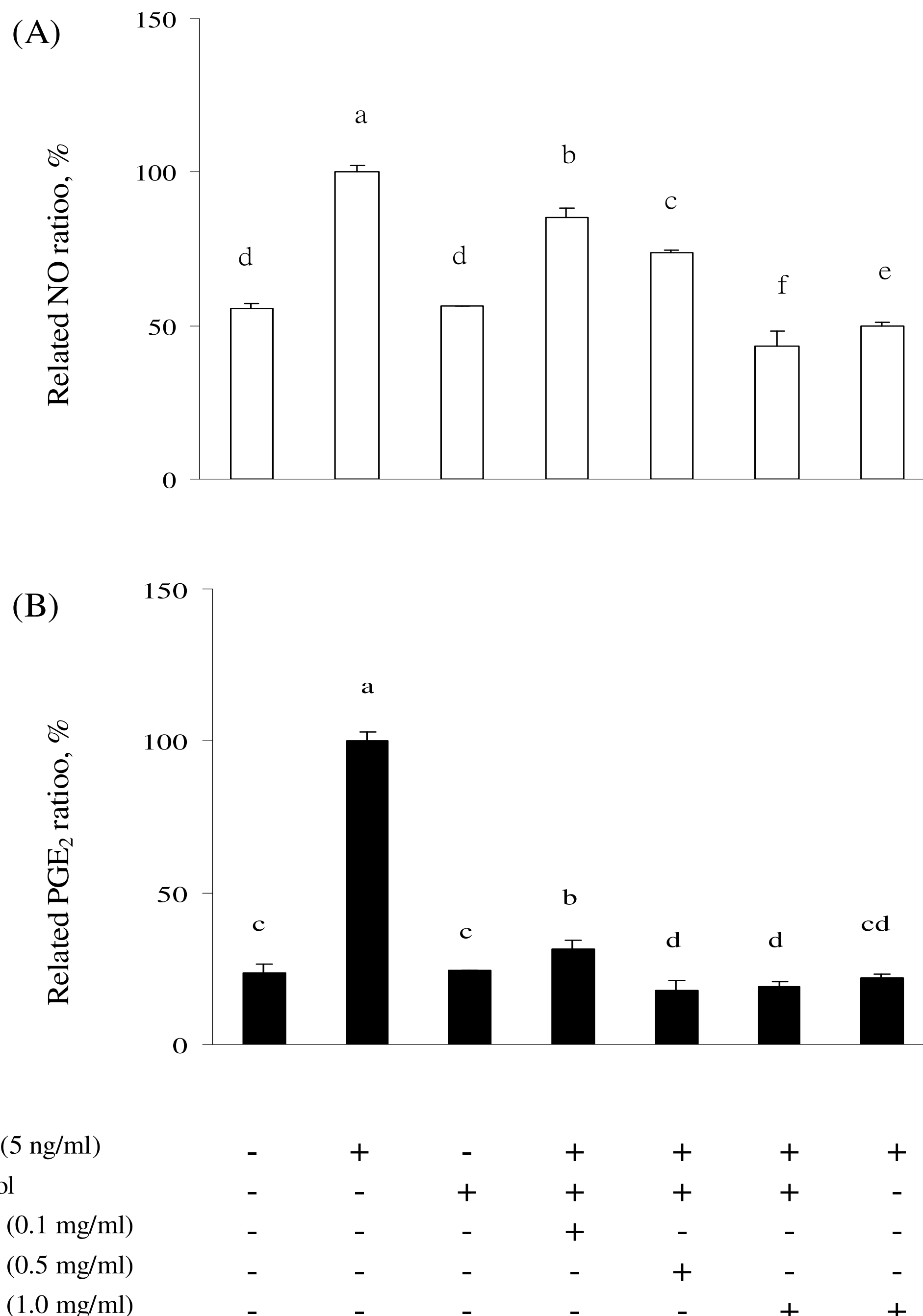


Figure 1. Effects of CTEE on NO (A) and PGE_2 (B) production in IL-1β-induced NRK-52E cells. NRK-52E cells were treated with IL-1β (5 ng/ml) alone or with CTEE (0.1, 0.5 or 1 mg/ml, respectively) for 18 h. Data are the mean ± S.D. from three or five independent experiments and are expressed as the percentage of the PBS vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).

with 0.5 ng/ml IL-1β and various concentrations of CTEE for 18 h. After collecting the culture media, the nitrite concentrations, reflecting the NO, within the media were assayed. IL-1β stimulation generated a noticeable accumulation of nitrite in the culture medium; however, CTEE significantly reduced the IL-1β-induced nitrite production in a dose-dependent manner (Figure 1A). Agreeing with the effect of CTEE on NO production, the CTEE also generated a remarkable decrease in PGE_2 in a dose-dependent manner (Figure 1B). When CTEE concentration was 1 mg/ml, the reduction percentage of IL-1β-induced NO and PGE_2 were 50.0 and 78.0%,

respectively. Which both inhibition of NO and PGE_2 correlated with the inhibition of iNOS and COX-2 activities, and both inhibition of NO and PGE_2 correlated with the inhibition of iNOS and COX-2 activities.

Effect of CTEE on IL-1β-induced mRNA and protein expression of iNOS and COX-2

For verifying whether the inhibitory effects of CTEE on NO and PGE_2 production originate from decreased messenger ribonucleic acid (mRNA) and protein expression of IL-1β-induced over expression of iNOS and COX-2 in NRK-52 cells, the RT-PCR and Western blot analysis have been executed. The expression of the iNOS and COX-2 mRNA and protein were significantly ($P < 0.05$) lower in unstimulated cells than those of in stimulated cells; however, these were extremely increased after IL-1β-induced treatment. CTEE exhibited a significant and dose-dependent inhibitory effect on mRNA and protein expression of iNOS and COX-2 in IL-1β-induced NRK-52E cells (Figure 2A and B). The low concentration (0.1 mg/ml) of CTEE could not significantly ($P > 0.05$) suppressed the mRNA expression; however, when CTEE concentration was 1 mg/ml, CTEE significantly ($P < 0.05$) decreased protein and mRNA expression of iNOS and COX-2 (by 90.0 and 64.0% for iNOS and by 87.0 and 85.0% for COX-2, respectively). The findings were consistent with the enhanced production of NO and PGE_2 shown in Figure 1.

Effect of CTEE on IL-1β-induced phosphorylation of IκBα

The phosphorylation of IκBα to p-IκBα can results in IκBα itself degradation and the release of NF-κB which then translocates to the nucleus. Accordingly, we inquired further whether CTEE could preclude the phosphorylation of IκBα induced by IL-1β. Because p-IκBα is a marker of NF-κB pathway activation, we have determined the expression of p-IκBα in this study. Figure 3 show that phosphorylation of IκBα was remarkably increased upon exposure to IL-1β alone, and that the phosphorylation was significantly ($P < 0.05$) inhibited in the presence of CTEE in a dose-dependent manner. And the inhibitory effect of CTEE on phosphorylation of IκBα was accounted for the following NF-κB nuclear translocation.

Effect of CTEE on IL-1β-induced nuclear translocation of NF-κB p65

To elucidate the mechanisms underlying the inhibition of iNOS and COX-2 expression in IL-1β-induced NRK-52E cells, we inspected the effects of CTEE on the NF-κB nuclear translocation. Because the NF-κB p65 is the major component of the activated NF-κB, we have evaluated the levels of NF-κB p65 in nuclear extracts by Western blotting analysis. CTEE inhibited IL-1β-induced nuclear translocation of NF-κB p65, in a dose-dependent manner (Figure 4). Taken together, these data suggest that the inhibitory effect of CTEE on the IL-1β-induced translocation of NF-κB p65 might be involved in the suppression of IκBα phosphorylation.

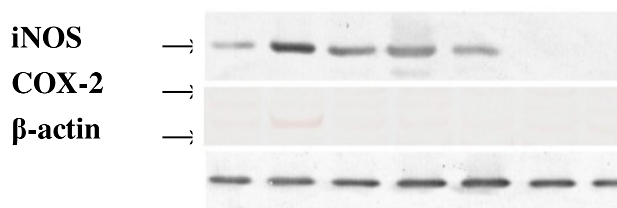
DISCUSSION

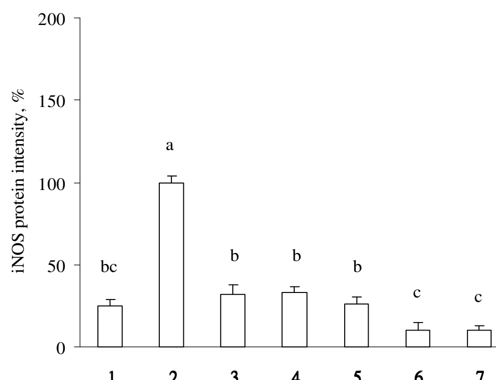
The inflammatory cytokine IL-1β plays as an immunoregulatory roles involved in many pathological processes. Several reports demonstrated that IL-1β stimulation significantly enhanced the production of NO and PGE_2 (Tamura et al., 2002; Wu et al., 2005) by enhancing the expression of COX-2 and iNOS (Wu and Guo, 2007); however, the stimulation was prevented by either transcriptional or translational blockers (Newton et al., 1997). The IL-1β was employed as a stimulator to induce inflammation response in NRK-52E cells in this study. The results indicated that IL-1β successfully induced inflammation response and increased the production of NO and PGE_2 in NRK-52E cells. As expected, the CTEE significantly reduced IL-1β induced NO and PGE_2 production in a dose-dependent manner in NRK-52E cells.

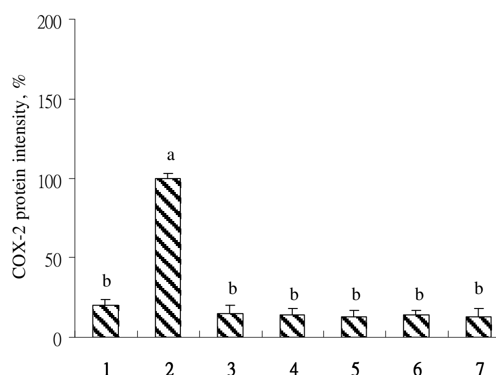
The NO production was regulated by constitutive NOS (cNOS) under normal physiological conditions (Nakagawa and Yokozawa, 2002); however, NO was also over-produced at inflamed sites by iNOS in inflammatory events (Kim et al., 1999). Therefore, regulation of iNOS in tissues is very important for the treatment of inflammation and tumorigenesis (Chan et al., 1998). On the other hand, COX-2, one of inducible enzymes also, is crucial in the inflammatory response, converts arachidonic acid to PGE_2 and is required in introducing and sustaining reactions during the inflammatory course (Surh et al., 2001). Park et al. (2004) noted that overproduction of PGE_2 mediated by COX-2 was connected to the development of inflammation and carcinogenesis. In this study, we found the expression of iNOS and COX-2 protein and mRNA were inhibited by CTEE, confirming the suppressive effect of CTEE on the NO and PGE_2 production. The present work also showed that the inhibitory effect of CTEE on IL-1β-induced iNOS and COX-2 expression may have resulted from the transcriptional inhibition of iNOS and COX-2 gene, respectively.

The present study demonstrates for the first analysis detecting NF-κB and p-IκBα expression in IL-1β-induced NRK-52E cells. Our data show that IκBα was phosphorylated and was degraded to p-IκBα and then the NF-κB complex was presented both in cytosol and nucleus. Because the phosphorylation and degradation of IκBα is necessary for NF-κB activation (Henkel et al., 1993)

(A)

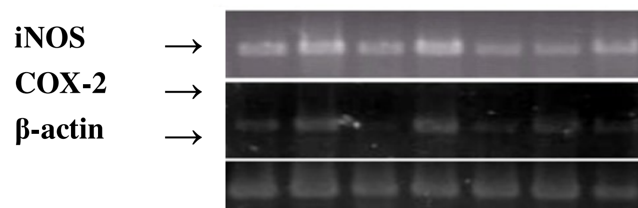


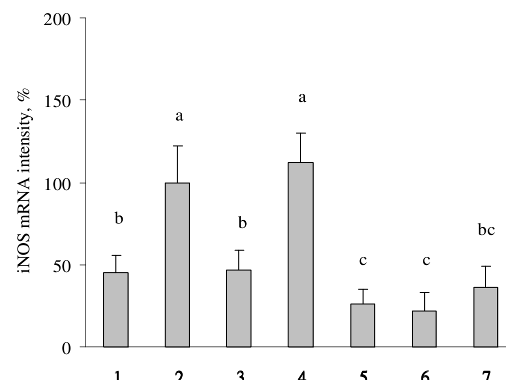


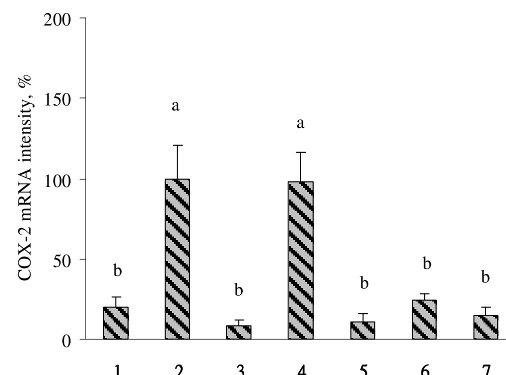


IL-1β (5 ng/ml)	-	+	-	+	+	+	+
Ethanol	-	-	+	+	+	+	-
CTEE (0.1 mg/ml)	-	-	-	+	-	-	-
CTEE (0.5 mg/ml)	-	-	-	-	+	-	-
CTEE (1.0 mg/ml)	-	-	-	-	-	+	+

(B)







IL-1β (5 ng/ml)	-	+	-	+	+	+	+
Ethanol	-	-	+	+	+	+	-
CTEE (0.1 mg/ml)	-	-	-	+	-	-	-
CTEE (0.5 mg/ml)	-	-	-	-	+	-	-
CTEE (1.0 mg/ml)	-	-	-	-	-	+	+

Figure 2. Effects of CTEE on iNOS and COX-2 protein (A) and mRNA (B) expression in NRK-52E cells. NRK-52E cells were treated with IL-1β (5 ng/ml) alone or with CTEE (0.1, 0.5 or 1 mg/ml, respectively) for 18 h. Protein extracts from cell pellets were subjected to SDS-PAGE followed by the Western blot analysis using anti-COX-2 and anti-iNOS antibodies. Total mRNAs were prepared from the cell pellets using TRIzol. The relative levels of mRNAs were assessed by RT-PCR. Results were normalized to β-actin. Data are the mean ± S.D. from three or five independent experiments and are expressed as the percentage of the PBS vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).

and NF-κB signaling pathway involved in the regulation of inflammatory responses (Silverman and Maniatis, 2001), herein, base on our results, we not only considered that IL-1β successfully induced the inflammation but also showed that CTEE blocked the NF-κB signaling pathway in IL-1β-induced NRK-52E cells. In terms of CTEE anti-inflammatory mechanism, we showed that CTEE exerted the inhibitory effect on the expression of iNOS and COX-2, as well as the production of NO and PGE_2; moreover, the inhibitory effect was accompanied by a reduced

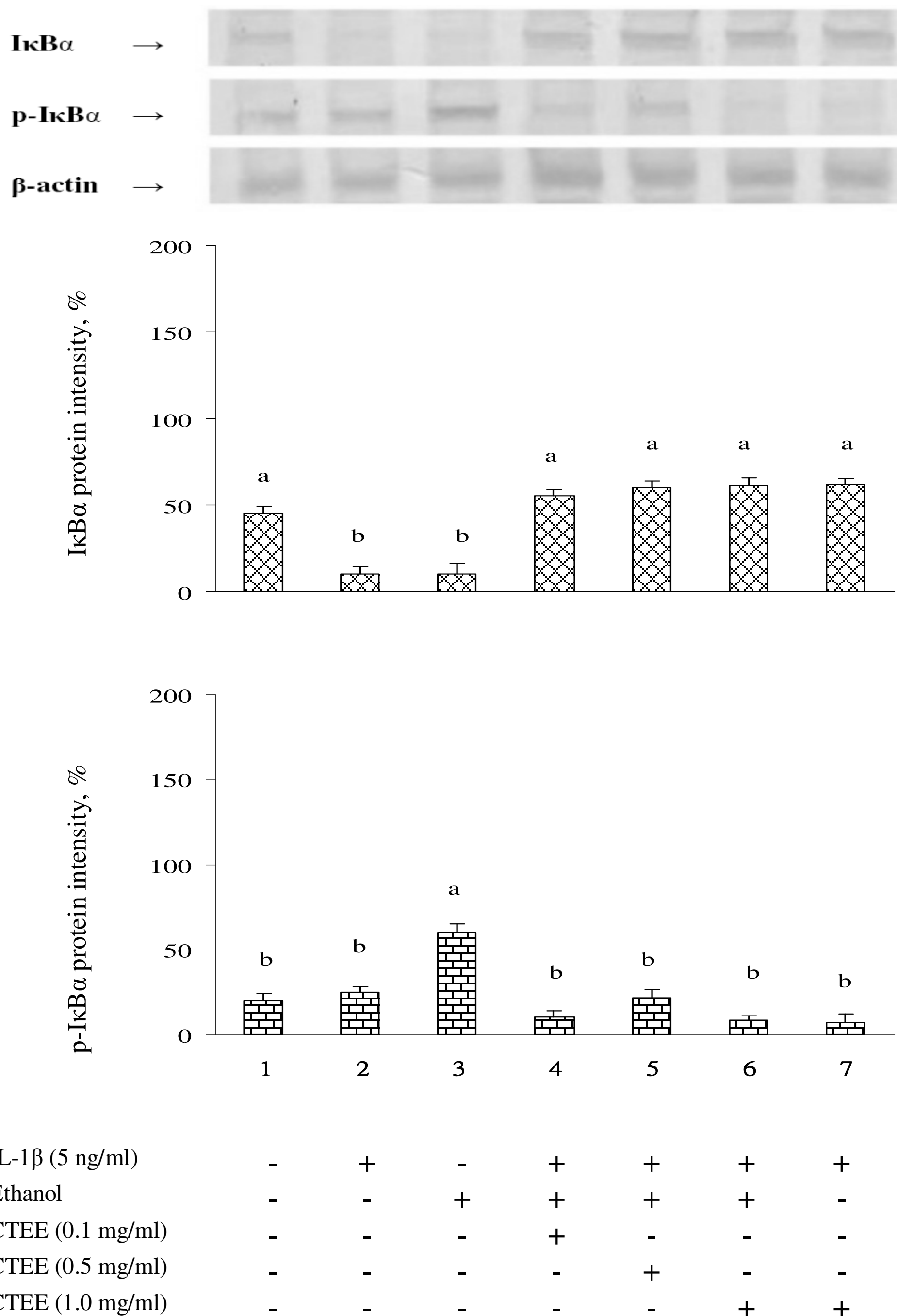
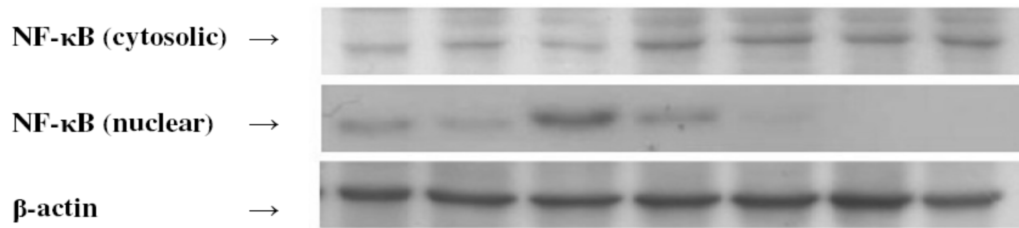
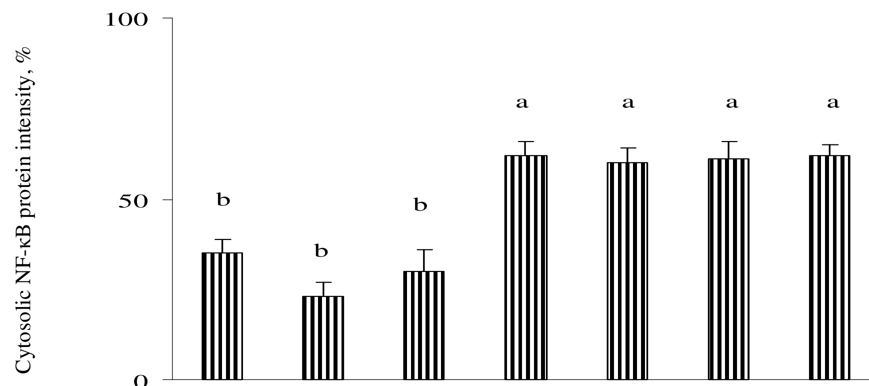
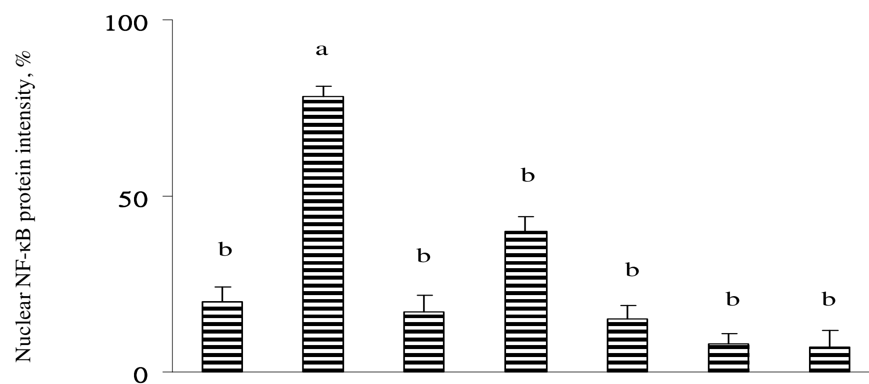


Figure 3. Effects of CTEE on IκBα and p-IκBα protein level on NRK-52E induced by IL-1β. NRK-52E cells were treated with IL-1β (5 ng/ml) alone or with CTEE (0.1, 0.5 or 1 mg/ml, respectively) for 18 h. Results were normalized to β-actin. Data are the mean ± SD from three or five independent experiments and are expressed as the percentage of the PBS vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).

phosphorylation of IκBα, confirming the inhibition resulted from the blockage of NF-κB pathway. Recently, one of the newly designed drugs for inflammation-related diseases was to design an inhibitor to block the NF-κB signaling pathways (Viatour et al., 2005). The *C. taiwanianum* Yamaz. might be a potential candidate as a new drug agent. The mechanism of anti-inflammatory effect of CTEE in IL-1β-induced NRK-52E cells was elucidated in this study, namely the suppression of NF-κB activation caused by CTEE accounted for the inhibition of the expression of iNOS and COX-2, as well as the following inhibition of production of NO and PGE_2.







IL-1β (5 ng/ml)	-	+	-	+	+	+	+
Ethanol	-	-	+	+	+	+	-
CTEE (0.1 mg/ml)	-	-	-	+	-	-	-
CTEE (0.5 mg/ml)	-	-	-	-	+	-	-
CTEE (1.0 mg/ml)	-	-	-	-	-	+	+

Figure 4. Effects of CTEE on cytosolic and nuclear NF-κB protein level on NRK-52E induced by IL-1β. NRK-52E cells were treated with IL-1β (5 ng/ml) alone or with CTEE (0.1, 0.5 or 1 mg/ml, respectively) for 18 h. Results were normalized to β-actin. Data are the mean ± SD from three or five independent experiments and are expressed as the percentage of the PBS vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).

Many flavonoids present in numerous dietary plants (Yoon et al., 2005) and medicine herbs (Huang et al., 1994) and showed anti-inflammatory activities through the inhibition of COX-2 and iNOS expression (Jung et al., 2007) as well as the suppression of NO production (Kim et al., 2005). Flavonoids including kaempferol and quercetin in *C taiwanianum* Yamaz. were identified (Chen et al., 1991; Lin et al., 1998). Consistent with these

previous reports, we also found the presence of quercetin in CTEE (our unpublished data). Quercetin is one of naturally occurring dietary flavonoids found in many plants and can down-regulate the iNOS protein expression in A549 human lung adenocarcinoma cells (Banerjee et al., 2002) and inhibits IL-1β-induced proliferation in human airway smoothmuscle cells (Kumar-Roiné et al., 2009) as well as attenuate NO production in IL-1β-induced hepatocytes (Martínez-Flórez et al., 2005). However, the anti-inflammatory compound(s) of C. *Taiwanianum* Yamaz. and/or their potential synergistic activation are unclear at present and must await future studies. These works are undertaken in our laboratory.

In conclusion, the suppression of NO and PGE_2 production by CTEE in NRK-52E cells treated with IL-1β could have result from the inhibition of protein expression and mRNA transcription of iNOS and COX-2, respectively. Moreover, the capacity of inhibition of NF-κB nuclear protein DNA binding activity and of IκBα phosphorylation may account, at least in part, for the anti-inflammatory mechanism of *C. taiwanianum* Yamaz.

Abbreviations

COX-2, Cyclooxygenase-2; **iNOS,** inducible nitric oxide synthase; **CTEE,** *Cynanchum taiwanianum* Yamazaki rhizome ethanolic extract; **IκBα,** I kappa B-alpha; **p-IκBα,** phospho-IκBαl; **NF-κB,** nuclear factor kappa B; **mRNA,** messenger ribonucleic acid; **NO**, nitric oxide; **PGE_2,** prostaglandin E2.

REFERENCES

Banerjee T, Van der Vliet A, Ziboh VA (2002). Downregulation of COX-2 and iNOS by amentoflavone and quercetin in A549 human lung adenocarcinoma cell line. PLEFA, 66: 485-492.

Chan MM, Hunag HI, Fenton MR, Fong D (1998). *In vivo* inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. Biochem. Pharmacol. 55: 1955-1962.

Chen ZS, Lai JS, Kuo YH (1991). The constituents of *Cynanchum taiwanianum*. J. Chin. Chem. Soc. 38: 393-396.

Coussens LM, Werb Z (2002). Inflammation and cancer. Nature, 420: 860-867.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982). Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal. Biochem. 126: 131-138.

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

Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y, Baeuerle PA (1993). Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature, 365:182-185.

Huang HC, Wang HR, Hsieh LM (1994). Antiproliferative effect of baicalein, a flavonoid from a Chinese herb, on vascular smooth muscle cell. Eur. J. Pharmacol. 251:91-93.

Jung CH, Kim JH, Hong MH, Seog HM, Oh SH, Lee PJ, Kim GJ, Kim HM, Um JY, Ko SG (2007). Phenolic-rich fraction from *Rhus verniciflua* Stokes (RVS) suppress inflammatory response via NF-κB and JNK pathway in lipopolysaccharide-induced RAW 264.7 macrophages. J. Ethnopharmacol. 110: 490-497.

Kim AR, Cho JY, Zou Y, Choi JS, Chung HY (2005). Flavonoids differentially modulate nitric oxide production pathways in lipopolysaccharide-activated RAW 264.7 cells. Arch. Pharm. Res. 28: 297-304.

Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem. Pharmacol. 58: 759-765.

Koo TH, Lee JH, Park YJ, Hong YS, Kim HS, Kim KW, Lee JJ (2001). A sesquiterpene lactone, costunolide from *Magnolia grandiflora*, inhibits NF-κB by targeting IκB phosphorylation. Planta Med. 67:103-107.

Kumar-Roiné S, Matsui M, Reybier K, Darius HT, Chinain M, Pauillac S, Laurent D (2009). Ability of certain plant extracts traditionally used to treat ciguatera fish poisoning to inhibit nitric oxide production in RAW 264.7 macrophages. J. Ethnopharmacol. 123: 369-377.

La Grutta S, Gagliardo R, Mirabella F, Pajno GB, Bonsignore G, Bousquet J, Bellia V, Vignola AM (2003). Clinical and biological heterogeneity in children with moderate asthma. Am. J. Respir. Crit. Care. Med. 167: 1490-1495.

Lii CK, Chen HW, Yun WT, Liu KL (2009). Suppressive effects of wild bitter gourd (*Momordica charantia* Linn. var. *abbreviata* ser.) fruit extracts on inflammatory responses in RAW 264.7 macrophages. J. Ethnopharmacol. 124: 227-233.

Lin CC, Yen MH, Wu YW, Xu GJ (1995). The histological and biological studies on *Cynanchum taiwanianum* proceeding of a symposium on development and utilization of resources of medicinal plants in Taiwan. TARI Special Publ. 48: 313-320.

Lin CN, Huang PL, Wang JJ, Day SW, Lin HC, Wang JP, Ko YL, Teng CM (1998). Stereochemistry and biological activities of constituents from *Cynanchum taiwanianum*. Biochim. Biophys. Acta (BBA)/General Subjects 1380: 115-122.

Martínez-Flórez S, Gutíerrez-Fernández B, Sánchez-Campos S, González-Gallegon J, Tuñón MJ (2005). Quercetin attenuates nuclear factor-κB activation and nitric oxide production in interleukin-1β-activated rat hepatocytes. J. Nutr. 135: 1359-1365.

Mordan LJ, Burnett TS, Zhang LX (1993). Inhibitors of endogenous nitrogen oxide formation block the promotion of neoplastic transformation in C3H 10T1/2 fibroblasts. Carcinogenesis, 14: 1555-1559.

Nakagawa T, Yokozawa T (2002). Direct scavenging of nitric oxide and superoxide by green tea. Food Chem. Toxicol. 40: 1745-1750.

Narumiya S, Sugimoto Y, Ushikubi F (1999). Prostanoid receptors: structures, properties, and functions. Physiol. Rev. 79: 1193-1226.

Newton R, Kuitert LM, Slater, DM, Adcock IM, Barnes PJ (1997). Cytokine induction of cytosolic phospholipase A2 and cyclooxygenase-2 mRNA is suppressed by glucocorticoids in human epithelial cells. Life Sci. 60: 67-78.

Newton R, Stevens DA, Hart LA, Lindsay M, Adcock IM, Barnes PJ (1997). Superinduction of COX-2 mRNA by cycloheximide and interleukin-1β involves increased transcription and correlates with increased NF-κB and JNK activation. FEBS Lett. 418: 135-138.

Park EJ, Min HY, Ahn YH, Bae CM, Pyee JH, Lee SK (2004). Synthesis and inhibitory effects of pinosylvin derivatives on prostaglandin E2 production in lipopolysaccharide-induced mouse macrophage cells. Bioorg. Med. Chem. Lett. 14: 5895-5898.

Silverman N, Maniatis T (2001). NF-kappa B signaling pathways in mammalian and insect innate immunity. Genes Dev. 15: 2321-2342.

Smith WL, DeWitt DL, Garavito RM (2000). Cyclooxygenases: structural, cellular, and molecular biology. Ann. Rev. Biochem. 69: 145-182.

Sripanidkulchai B, Junlatata J, Wara-aswapatia N, Hormdeea D (2009). Anti-inflammatory effect of *Streblus asper* leaf extract in rats and itsmodulation on inflammation-associated genes expression in RAW 264.7 macrophage cells. J. Ethnopharmacol. 124: 566-570.

Stylianou E, Saklatvala J (1998). Interleukin-1. Int. J. Biochem. Cell Biol. 30: 1075-1079.

Surh YJ, Chun KS, Cha HH, Han SS, Keum Y, Park K, Lee SS (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. Mutat. Res. 480-481: 243-268.

Tamura M, Sebastian S, Yang S, Gurates B, Fang Z, Bulun SE (2002). Interleukin-1β elevates cyclooxygenase-2 protein level and enzyme its mRNA stability in human endometrial stromal cells: an effect

mediated by extracellularly regulated kinases 1 and 2. J. Clin. Endocrinol. Metab. 87: 3263-3273.
Viatour P, Merville MP, Bours V, Chariot A (2005). Phosphorylation of NF-κB and IkB proteins:implications in cancer and inflammation. Trends Biochem. Sci. 30: 43-52.
Vinayagamoorthi R, Koner BC, Kavitha S, Nandakumar DN, Padma Priya P, Goswami K (2005). Potentiation of humoral immune response and activation of NF-κB pathway in lymphocytes in experimentally induced hyperthyroid rats. Cell Immunol. 238: 56-60.
Wu MH, Wang CA, Lin CC, Chen LC, Chang WC, Tsai SJ (2005). Distinct regulation of cyclooxygenase-2 by interleukin-1β in normal and endometriotic stromal cells. J. Clin. Endocrinol. Metab. 90: 286-295.
Wu Y, Guo SW (2007). Suppression of IL-1β-induced COX-2 expression by trichostatin A (TSA) in human endometrial stromal cells. Eur. J. Obstet. Gynecol. Reprod. Biol. 135: 88-93.
Yoon JH, Baek SJ (2005). Molecular targets of dietary polyphenols with anti-inflammatory properties. Yonsei Med. J. 46: 585-596.
Yin MJ, Yamamoto Y, Gaynor RB (1998). The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature, 396: 77-80.