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Aqueous extract isolated from *Platycodon grandiflorum* elicits the release of nitric oxide and tumor necrosis factor-α from murine macrophages

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Abstract

Herbal medicines are increasingly being utilized to treat a wide variety of disease processes. Aqueous extract from the root of *Platycodon grandiflorum* A. DC (Campanulaceae), Changkil (CK), is reported to have antitumor and immunomodulatory activities; however, the mechanism underlying its therapeutic effect is not known. In the present study we examined the effects of CK on the release of nitric oxide (NO) and tumor necrosis factor-α (TNF-α), and on the gene expression of iNOS and TNF-α in mouse macrophages. CK elicited a dose-dependent increase in NO and TNF-α production in cultured macrophages. CK significantly affected secretion at concentrations of more than 5 μg/ml, and its maximum effect was at concentration of 100 μg/ml. Reverse transcription polymerase chain reaction showed that increases in NO and TNF-α secretion were due to an increase in inducible NO synthase mRNA and TNF-α mRNA, respectively. Transient expression assays with NF-κB binding sites linked to the luciferase gene revealed that CK-induced increase of inducible NO synthase mRNA and TNF-α mRNA were mediated by the NF-κB transcription factor complex. These results demonstrate that CK stimulates NO and TNF-α release and is able to upregulate iNOS and TNF-α expression through NF-κB transactivation and this may be a mechanism whereby this herbal medicine elicits its therapeutic effects.

Keywords: *Platycodon grandiflorum*; Macrophages; Nitric oxide; Tumor necrosis factor-α

1. Introduction

Macrophages play a significant role in host defense mechanisms. When activated, they inhibit the growth of a wide variety of tumor cells and microor-

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ganisms. Nitric oxide (NO), a free-radical gas, is synthesized by nitric oxide synthase (NOS) [1] and mediates diverse functions, including vasodilatation, neurotransmission, the inhibition of platelet aggregation, immunoresponses and the inhibition of extracellular matrix production [2]. NO is identified as the major effector molecule in the destruction of tumor cells by activated macrophages [3]. Involvement of NO during nonspecific host defense, macrophage-mediated killing, or inhibition of the proliferation of microorganisms and tumor cells both in vitro [4] and in vivo [5] has been previously demonstrated. Furthermore, several studies have demonstrated that activated macrophages produce NO, and that reactive nitrogen intermediates are believed to play a significant role in tumoricidal and microbicidal activities [6]. Increased cytotoxic activity of LPS/IFN-γ-treated primary macrophages on guinea pig L10 hepatoma and mouse L1210 lymphoma cells via NO was reported by Stuehr and Nathan [4]. Such NO-mediated tumoricidal activity is induced by DNA damage and leads to apoptotic cell death [7]. The administration of NOS inhibitors to mice has promoted the growth of several transplantable tumors [5], and melanoma cells transfected with iNOS cDNA did not proliferate and metastasize well [8]. Tumor necrosis factor-α (TNF-α) is produced by activated macrophages, fibroblasts, and many different types of cells. TNF-α has also been recognized and well characterized as an important host defense molecule that affects tumor cells [3,9–11]. In macrophages, nuclear factor κB (NF-κB), in cooperation with other transcription factors, coordinates the expression of genes encoding both iNOS and TNF-α. NF-κB plays a critical role in the activation of immune cells by upregulating the expression of many cytokines essential to the immune response [12].

Epidemiological studies have indicated that almost all cases of cancer are associated with environmental factors, including food [13]. Herbs have recently become attractive as health-beneficial foods (physiologically functional foods) and as a source material for the development of drugs. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, with relatively little knowledge regarding their modes of action. Immune responses are modulated by various types of agents, including bacterial, fungal, plant and synthetic products. A number of β-glucans show strong immunostimulation and antitumor activities and polysaccharide isolated from the mushroom *Phellinus linteus* also stimulate immunity [14].

Platycodi Radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae), commonly known as Doraji (Chinese drug, 'Jiegeng', and Japanese name, 'Kikyo'), has been used as a traditional oriental medicine [15,16]. Extracts from the roots of *P. grandiflorum* have been reported to have a wide range of health benefits. In Korea, the root of *P. grandiflorum* (4 years old) has been used as a food and employed as a folk remedy for diseases of adulthood, such as bronchitis, asthma, pulmonary tuberculosis, hyperlipidemia, diabetes, inflammatory diseases, and as a sedative and analgesic [15–17]. Some studies on its chemical [18,19] and immunopharmacological effects [15,16,20–22] have been performed; however, little is known about its immunostimulating effects or its mechanisms. Recently, it has been observed that the root of *P. grandiflorum* (22 years old) had proved beneficial to obese patients with adult-onset diabetes mellitus and lung cancer (unpublished data), and that dietary *P. grandiflorum* has a beneficial effect on preventing hypercholesterolemia and hyperlipidemia [17].

In a previous study, we showed that the Changkil (CK), aqueous extract from the root of *P. grandiflorum*, cultivated for more than 20 years,[1] inhibited the growth of various transplantable tumors in experimental animals and increase the survival rate [23] and, thus, it is believed that this preparation may well have important antitumor and immune stimulating properties. CK is considered to exert its antitumor activity through potentiation of the host animals' defense system, rather than via direct inhibition of tumor cell growth [23]. It has also been reported that the methanolic extract of *P. grandiflorum* promoted the phagocytic activity of the mouse reticuloendothelial system and peritoneal macrophage [21].

Although CK is thought to augment immune response via the modulation of the function of phago-

[1] S.H. Lee, Patent on the method of cultivating the perennial balloonflower , 1991 (patent no. 045971, Korea).

cytes, such as macrophages, the precise mechanism for the augmentation of cell-mediated immunity remains to be elucidated. Due to our previous report upon the benefits of CK on antitumor activity [23], in this study, we tested the hypothesis that one potential pharmacological mode of CK action is via the release of NO and TNF-α from macrophages. As far as we know, there is no evidence that CK induces secretion and gene expression of NO and TNF-α in murine macrophages. After the administration of CK, the production of NO and TNF-α, the expression of inducible NO synthase and TNF-α, and NF-κB activation were elevated compared with the control. These results suggest that the CK may induce Th immune responses.

2. Materials and methods

2.1. Plant material

Aqueous extract (CK) from the root of *P. grandiflorum* (22 years old), supplied by Jangsaeng Doraji, Chinju, South Korea, was prepared as follows: powder roots was added to distilled water (5 ml/g) and the mixture maintained at 90°C for 10 h, cooled to room temperature, then filtered and lyophilized. The yield of lyophilized residue corresponded to 33.5% (33.5 g of residue for each 100 g of original dry roots). The pale-yellow extract was dissolved directly in distilled water. The composition of the root of *P. grandiflorum* was shown in previous study [17].

2.2. Chemicals

Chemicals and cell-culture materials were obtained from the following sources: oligo(dT) 18 primers, Moloney leukaemia virus (M-MLV) reverse transcriptase (RT), LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and penicillin–streptomycin solution (Life Technologies); pGL3-4KB-Luc and luciferase assay system (Promega); pCMV-β-gal (Clonetech); *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) and Polymyxin B sulfate (Sigma); AmpliTaq® DNA polymerase (Perkin-Elmer); dNTP solutions and ribonuclease (RNAse) inhibitor (Takara Technologies); goat monoclonal antibody against murine TNF-α, biotinylated goat polyclonal antibody against murine TNF-α, recombinant murine TNF-α, horseradish peroxidase streptavidin, 3,3′,5,5′-tetramethylbenzidine, and H_2O_2 (R & D Systems). Other chemicals were of the highest commercial grade available. Polymerase chain reaction (PCR) oligonucleotide primer pairs were custom synthesized by Bioneer (Korea).

2.3. Animals

Specific pathogen free-BALB/C mice (female, 5–7 weeks old) were obtained from KRIBB (South Korea). Mice were housed under normal laboratory conditions, i.e., at 21–24°C and 40–60% relative humidity with a 12 h light/dark cycle with free access to standard rodent food and water.

2.4. Preparation of peritoneal macrophages and cell cultures

Peritoneal macrophages were isolated from mice that had been injected intraperitoneally with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on a flat-bottom culture plate and then incubated for 2 h at 37°C in a 5% CO_2 humidified incubator. After removing the nonadherent cells, the monolayered macrophages were treated with sample solution.

RAW 264.7 cells, mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator.

2.5. Endotoxin assay

To assay CK for the presence of gram-negative bacterial endotoxin, LPS, an E-Toxate test (Limulus Amebocyte Lysate; Sigma) was used, according to the manufacturer's instructions.

2.6. Nitrite assay

Peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) prepared as above were cultured in 48-well plates. After 24 h incubation, NO synthesis was determined by assaying culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen. Briefly, 100 μl of medium from the 48-well plates was incubated with an equal volume of Griess reagent for 15 min at room temperature. The absorbance at 550 nm was measured using a FL600 (Bio-Tek) microplate reader. Nitrite concentration was determined using dilutions of sodium nitrite in culture medium as standards. By adding CK to standard solutions of sodium nitrite, it was confirmed that CK did not interfere with the nitrite assay.

2.7. Immunoassay of TNF-α

For immunoassay of TNF-α, peritoneal macrophages or RAW 264.7 cells were cultured at a density of 2×10^6 cells/ml for 6 h in 24-well plates. TNF-α production was quantified by sandwich immunoassays, and measured with microtiter plates coated with the goat monoclonal antibody against murine TNF-α, and the biotinylated goat polyclonal antibody against murine TNF-α, horseradish peroxidase streptavidin, 3,3′,5,5′-tetramethylbenzidine and H_2O_2. The reaction was stopped with 1 M H_2SO_4 and plates were read at 450 nm in a FL600 (Bio-Tek) microplate reader. Recombinant murine TNF-α was used as a standard.

2.8. RNA extraction

For RNA extraction, the cells (1×10^6/ml) were cultured with CK. After 6 h incubation, the cells were lysed in 4 M guanidinium thiocyanate solution. Total RNA was extracted according to the single-step method previously described by Chomczynski and Sacchi [24]. Before use, the integrity and purity of the RNA samples were checked by electrophoresis. Visualization of the 28S and 18S rRNA species as discrete bands, and the absence of any high molecular weight species were taken to indicate that the samples were intact and free of genomic DNA contamination.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

The reverse transcription reactions (20 μl total volume) were carried out in 5 mM $MgCl_2$, PCR buffer (50 mM KCl; 10 mM Tris–HCl pH 8.3), using 1 mM dNTPs, 1.75 units/μl RNAse inhibitor, 2.5 units/μl M-MLV reverse transcriptase, 25 μg/μl oligo(dT) primers and 25 ng total RNA. All the tubes were incubated at room temperature for 10 min before undergoing the following reverse transcription protocol in a thermal cycler: 42°C for 60 min; 94°C for 3 min. After the generation of the first strand cDNAs, the tubes were placed on ice for 5 min prior to either being stored at −20°C or used for the polymerase chain amplification step. The PCR reactions (100 μl total volume) were carried out in 2 mM $MgCl_2$, PCR buffer, using 2.5 units AmpliTaq® DNA polymerase, 5 μg/ml sense and antisense primer pair and a 20 μl reverse transcription volume. The sense and antisense primer sequences are: iNOS: 5′-CAT GGC TTG CCC CTG GAA GTT TCT CTT CAA AG-3′, 5′-GCA GCA TCC CCT CTG ATG GTG CCA TCG-3′; TNF-α: 5′-ATG AGC ACA GAA AGC ATG ATC CGC-3′, 5′-CCA AAG TAG ACC TGC CCG GAC TC-3′; and β-actin: 5′-CCT CTA TGC CAA CAC AGT-3′, 5′-AGC CAC CAA TCC ACA CAG-3′. These primer sets yielded PCR products of 754, 692 and 153 bp for iNOS, TNF-α and β-actin, respectively. PCR (35 cycles for iNOS and TNF-α cDNAs and 25 cycles for β-actin cDNA) was performed using the GeneAmp PCR System 2400 (Perkin-Elmer). An initial PCR cycle of 94°C for 5 min, 60°C for 5 min and 72°C for 90 s, was carried out by the following temperature profile: denaturation, 94°C for 45 s; primer annealing, 60°C for 45 s; and primer extension, 72°C for 90 s. A final cycle of 94°C for 45 s, 60°C for 45 s and 72°C for 10 min was carried out.

When the PCR reactions were complete 0.05 or 0.2 volumes of the reaction mix, depending upon the expected amount of product, were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. The gel images were captured on a Gel Doc Image Analysis System (Kodak) and the yield of PCR products was normalized to β-actin after quantitative estimation using NIH Image software (Bethesda, MD). The

relative expression level was arbitrarily set at 1.0 in the control group. Prior to analysis, the PCR product band intensities were checked to ensure that they had not reached saturation.

2.10. Transfection and luciferase and β-galactosidase assays

RAW 264.7 cells (5×10^5 cells/ml) were plated in each well of a 12-well plate, and 12 h later transiently cotransfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of pGL3-4κB-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to cells. After 18 h, cells were treated with CK or LPS for 6 h, and then cells were lysed with lysis buffer (0.5 M HEPES, pH. 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton X-100). Luciferase activity was determined using the luciferase assay system (Promega) according to the manufacturer's instructions using a luminometer. The β-galactosidase assay was carried out in a total volume of 250 μl of the assay buffer containing 0.12 M Na_2HPO_4, 0.08 M NaH_2PO_4, 0.02 M KCl, 0.002 M $MgCl_2$, 0.1 M β-mercaptoethanol, 50 μg of *o*-nitrophenyl-β-galactoside, and 100 μg of cell extract. Luciferase activity was normalized using β-galactosidase activity and was expressed as relative to the activity detected with the controls.

2.11. Statistical analysis

All experiments were repeated at least three times. Student's *t*-test was used to assess the statistical significance of differences. A confidence level of < 0.05 was considered significant.

3. Results

3.1. Effects of CK on NO and TNF-α production

Since CK is known to activate macrophage and have antitumor activity [21–23], we studied the effects of CK on the production of NO and TNF-α and gene expression in murine peritoneal macrophages and RAW 264.7, a murine macrophage cell line. NO production-induced CK was assessed using the Griess reaction. NO was secreted only after a long period of time, no significant effect was observed after 6 h incubation; however, significant NO secretion was observed after 24 h (data not shown). A lag phase of about 6 h followed by a linear increase in NO level, which continued for 48 h, was observed in mouse macrophages and RAW 264.7 cells, a mouse macrophage cell line. The effect of the CK increased gradually after between 12 and 72 h incubation (data not shown). Therefore, the effect of CK on NO secretion was assayed after 24 h in subsequent experiments. The basal level of NO in untreated peritoneal macrophages was less than 2 μM (Fig. 1). CK showed a significant effect on NO production from 5 μg/ml; however, no significant effect was observed at a concentration of 1 μg/ml. The effect of CK gradually increased between concentrations of 5 and 100 μg/ml, and a plateau was reached at 100 μg/ml, as shown in Fig. 1. Upon CK stimulation, NO synthesis by peritoneal macrophages increased in a dose-dependent manner. The potent macrophage activator LPS (0.5 μg/ml) increased nitrite synthesis compared to the control. Consistent with these findings, CK also induced nitrite generation in a dose-dependent manner in the mouse macrophage cell line, RAW 264.7 cells (Fig. 1). Based on these results and the relationship between NO and the cytolytic function of macrophages against a variety of tumors [5,25], we suggest that the antitumor effect of CK [23] might be mediated in part through the activation of NO generation.

When macrophages were cultured in the presence of the CK, TNF-α was rapidly secreted. The concentration of TNF-α almost plateaued after 6 h incubation, and was constant between 6 and 24 h (data not shown). The maximum concentration of TNF-α in the culture medium was observed after 6 h of incubation (data not shown). Therefore, the media after 6 h incubation were used for the TNF-α assay in subsequent experiments. As with the NO assay, there were significant increases in TNF-α secretions at CK concentrations greater than 5 μg/ml; however, no significant effect was observed at concentrations below 1 μg/ml, as shown in Fig. 2. Also, TNF-α production was significantly enhanced by LPS.

Macrophages can be induced to produce NO and TNF-α by LPS, endotoxins, or cytokines [26]. To

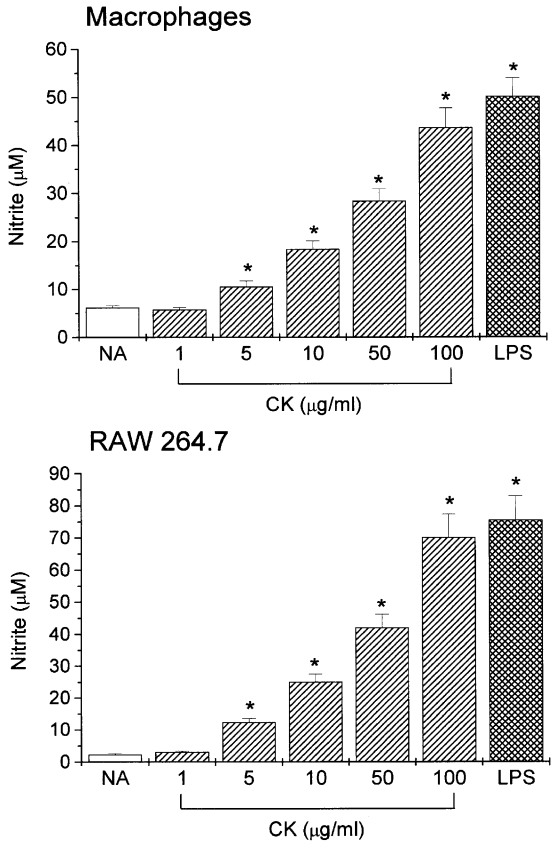

Fig. 1. Effects of CK on NO production. Murine peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured for 24 h in the presence of media alone, with the indicated concentrations of CK, or with LPS (0.5 μg/ml). NO production was determined by measuring the accumulation of nitrite in the incubation medium. Each bar shows the mean $\pm$ S.D. of four independent experiments, performed in triplicate. $^{*}P <$ 0.05, significantly different from the control.

confirm, that the ability of CK to induce NO and TNF-α could not be attributed to LPS contamination, the CK was tested for the presence of contaminating LPS using the *Limulus* amoebocyte lysate test. The level of LPS in CK was below the detection limit, which was typically below 12.5 pg/ml (data not shown). Polymyxin B sulfate has been used as a LPS inhibitor in macrophage cultures [27], and although CK contained no detectable activity in the *Limulus* amoebocyte lysate assay, in order to further check for possible LPS contamination in CK, polymyxin B (PMB; 10 μg/ml) was added to the cell cultures treated with the CK (100 μg/ml). As shown in Table 1, polymyxin B effectively inhibited NO and TNF-α secretion induced by 0.5 μg/ml LPS, but had no effect on the CK. This result demonstrated that production of NO and TNF-α by CK was unlikely to have resulted from LPS contamination in the CK.

3.2. Effects of CK on iNOS and TNF-α gene expression

As described above, CK-induced macrophage secretion of NO and TNF-α. In order to determine

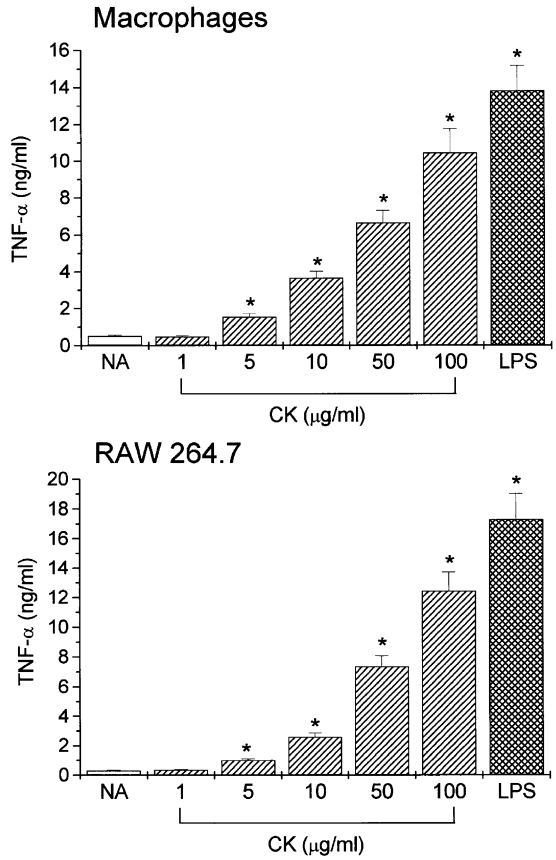

Fig. 2. Effect of CK on TNF-α secretion. Murine peritoneal macrophages (2×10^6 cells/ml) or RAW 264.7 cells (2×10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of CK, or with LPS (0.5 μg/ml). The amount of TNF-α released into the culture medium was measured by immunoassay. Each bar shows the mean $\pm$ S.D. of three independent experiments, performed in triplicate. $^{*}P < 0.05$, significantly different from the control.

Table 1
Effects of polymyxin B on NO and TNF-α secretion by CK and LPS

Treatment[a]	Nitrite (μM)[b]	TNF-α (ng/ml)[c]
Control	$2.47 \pm 0.43^{*}$	$0.51 \pm 0.04^{*}$
CK	69.47 ± 6.49	10.25 ± 1.01
CK + polymyxin B	62.17 ± 4.32	9.34 ± 0.92
LPS	78.21 ± 2.38	14.25 ± 1.41
LPS + polymyxin B	$9.39 \pm 0.95^{*}$	$3.04 \pm 0.03^{*}$

[a] RAW 264.7 cells (5×10^5 cells/ml for nitrite assay, and 2×10^6 cells/ml for TNF-α immunoassay) cultured with CK (100 μg/ml) or LPS (0.5 μg/ml), in the presence or absence of polymyxin B (10 μg/ml).

[b] Supernatants were harvested 24 h later and assayed for NO.

[c] Supernatants were harvested 6 h later and assayed for TNF-α. Values are mean $\pm$ S.D. of three individual experiments, performed in triplicate.

$^{*}P < 0.05$, significantly different from the LPS.

whether CK regulates NO and TNF-α secretion at the mRNA level, a reverse transcription-polymerase chain reaction (RT-PCR) assay was carried out. The intensity of the iNOS and TNF-α mRNA band increased after 2 h of incubation and a strong band was observed at 6 h (data not shown). Therefore, cells treated with CK for 6 h were used to assay of iNOS and TNF-α gene expression in subsequent experiments. RAW 264.7 cells were cultured with CK for 4 h and the mRNA expression of iNOS and TNF-α were determined. Total RNA extracted from cells that were cultured with different concentrations of CK was reverse-transcribed and amplified with iNOS or TNF-α-specific primers, as described in Materials and Methods. LPS (0.5 μg/ml), an immunostimulating agent, was used as a positive control. Consistent with the results obtained from the NO assay, iNOS mRNA levels were markedly increased by CK treatment (Fig. 3). This result indicates that the CK upregulated, in a dose-dependent manner, NO accumulation in macrophages. Therefore, we believe that the increased NO production by CK is regulated through transcriptional activation. Under the same treatment conditions, the TNF-α gene expression marker of macrophage activation was also examined, and CK was found to significantly enhance the expression levels of the TNF-α gene (Fig. 3). This result is consisted with that obtained from the immunoassay of TNF-α in

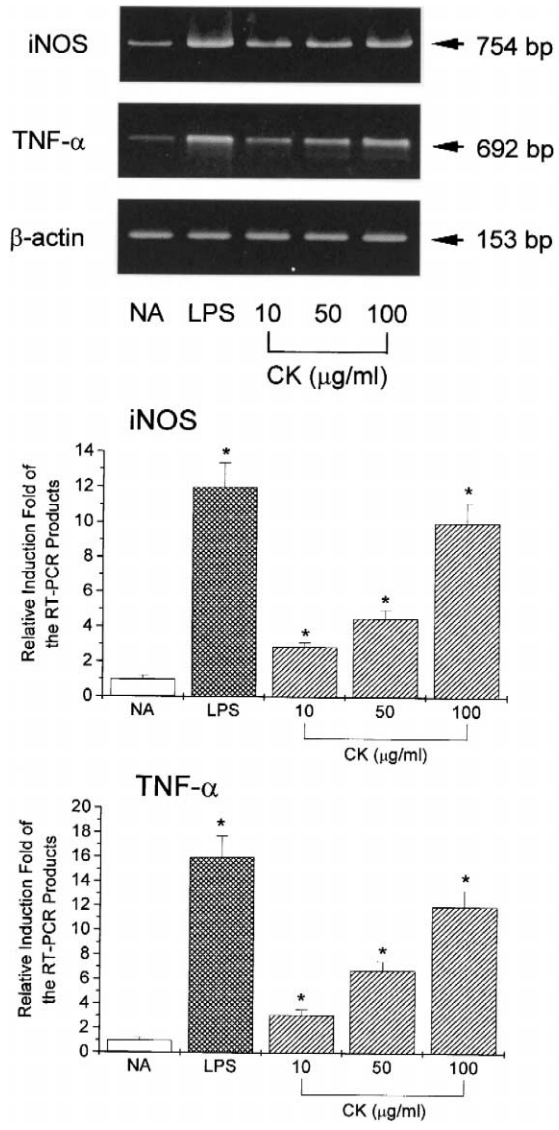

Fig. 3. Effects of CK on iNOS and TNF-α mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of CK, or with LPS (0.5 μg/ml). Cells were lysed and total RNA was prepared for RT-PCR analysis of gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide as described in Materials and Methods. One of three representative experiments is shown. The ratio of RT-PCR product of iNOS or TNF-α to β-actin was determined as described in Materials and Methods. Induction old is represented as the mean $\pm$ S.D. values of three separate experiments. $^{*}P < 0.05$, significantly different from the control.

macrophages and indicates that the CK also upregulates TNF-α accumulation in a dose-dependent manner.

3.3. Effects of CK on NF-κB-dependent gene expression

It has been reported that NF-κB plays a critical role in the expression of iNOS and TNF-α [12]. To further investigate the role of CK on iNOS and TNF-α gene expression, the effect of CK on NF-κB-dependent gene expression was assessed using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing 4 copies of the NF-κB binding sites and the luciferase activities were measured. LPS (0.5 μg/ml), an immunostimulating agent, was used as a positive control. Nearly a sevenfold increase in luciferase activity was observed when cells were stimulated with LPS as compared to unstimulated control cells. Similar to the results for NO and TNF-α production and iNOS and TNF-α mRNA level, CK significantly increased luciferase activities in a dose-dependent manner (Fig. 4). This result indicates that CK upregulates iNOS and TNF-α gene expression through NF-κB transactivation.

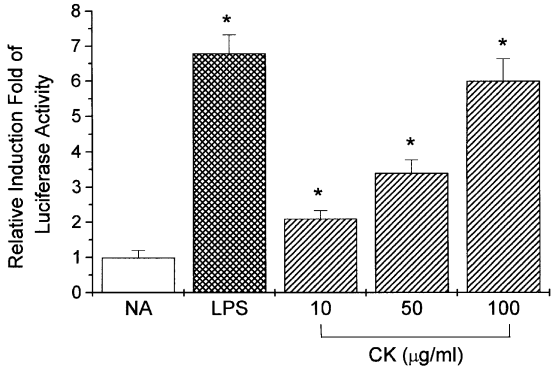

Fig. 4. Effects of CK on NF-κB-dependent luciferase gene expression. RAW 264.7 cells (5×10^5 cells/ml) were transiently cotransfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, cells were treated with the indicated concentrations of CK or LPS (0.5 μg/ml) for 6 h. Cells were harvested, and luciferase and β-galactosidase activities were determined as described in Materials and Methods. The luciferase activities were expressed as relative to that seen with the control (untreated cells). Each bar shows the mean $\pm$ S.D. of three independent experiments, performed in triplicate. $^{*}P < 0.05$, significantly different from the control.

4. Discussion

This is the first report that describes the induction of production and the gene expression of NO and TNF-α by the aqueous extract of the root of *P. grandiflorum* in macrophages. By administration of CK, the production of NO and TNF-α were augmented in treated murine peritoneal macrophages and RAW 264.7 cells, a mouse macrophage cell line. CK had a significant effect on NO and TNF-α secretion at levels above 5 μg/ml (Figs. 1 and 2). These results suggest that the secretion of NO and TNF-α is regulated by the same mechanism, or that TNF-α, which is produced first, induces NO secretion via an autocrine or paracrine system. TNF-α is the first compound of the TNF-α and NO series to be secreted by macrophages [28]. Thus, TNF-α is involved in the early phase of the cytokine cascade and induces NO production. The expression levels of iNOS and TNF-α genes were augmented in the treated RAW 264.7 cells (Fig. 3). NF-κB, a member of the Rel family, is a common regulatory element in the promoter region of many cytokines. In activated macrophages, NF-κB in synergy with other transcriptional activators plays a central role in coordinating the expression of genes encoding iNOS, TNF-α, IL-1, and IL-6 [12]. Consistent with data for both NO and TNF-α production and iNOS and TNF-α mRNA level, in the present study, CK increased NF-κB-dependent gene expression (Fig. 4). This results indicating that upregulation of these gene expression by CK is mediated in the activation of NF-κB.

NO is involved in the killing or inhibition of the proliferation of microorganisms, destruction of tumor cells by activated macrophages, and nonspecific host defense [4–6,25]. Furthermore, recent studies have demonstrated that murine macrophages stimulated with TNF-α [6] produce NO via expression of the inducible NOS gene [1], and it is believed that the reactive nitrogen intermediates so induced play a significant role in tumoricidal and microbicidal activities [6]. TNF-α, an endogeneous factor with tumor-selective cytotoxicity, has been recognized as an important host defense molecule that affects tumor cells. Moreover, the induction of NO and TNF-α production and gene expression by activated macrophages can lead to cytostatic and cytotoxic

activities on malignant cells [3,4,9–11,25]. Because of the pivotal role of NO and TNF-α in the antimicrobial and tumoricidal activities of macrophages, a significant effort has focused on developing therapeutic agents that regulate NO production and TNF-α secretion [29]. Therefore, the CK-stimulated release of NO and TNF-α may contribute to the antitumorigenic properties of *P. grandiflorum*. These results raise the possibility that the CK may induce Th immune responses.

It has been suggested that there are many immunomodulating cytokines and cytokine receptors in animals. However, not all immunomodulating cytokines are activated by antitumor drugs in vitro. It has been reported that the mycelia of *Coriolus versicolor*-induced gene expression in human peripheral blood mononuclear cells for IL-1β, IL-6, IL-8, TNF-α, etc. [30]. Leung et al. [31] reported that *Flammulina uelutipes* exhibits potent immunomodulatory and antitumor activity, and it has been demonstrated that this effect of *F. uelutipes* is not due to the direct killing of tumor cells, which suggests that the antitumor activity of *F. uelutipes* is mediated by a host immune-mediated system—similar to the mode of action of cytokines [31]. Also, Ito et al. [32] reported that *Aspracus blatei* increases the levels of NK cells and activated macrophages in vivo. Moreover, it was demonstrated that the mechanism of the antitumor effect of *A. blatei* seems to be mediated by the activation of NK cells and macrophages. We also found, using flow cytometry, that an intraperitoneal injection of CK increases the number of NK cells in blood lymphocyte [23].

We recently reported that the CK showed antitumor activity against the solid type of Sarcoma 180, but did not inhibit the growth of various cancer cell lines in vitro [23]. Therefore, the antitumor activity of the CK is likely to be mediated by potentiation of the host defense system in animals rather than by direct inhibition of tumor cell growth. Nagao et al. [22] reported that *P. grandiflorum* inhibited the growth of on intraperitoneally or intramuscularly transplanted Ehrlich ascites carcinoma in mice. The cytotoxicity of tumor cells is dependent on the activation of macrophages, and the expression of these mechanisms strongly correlates with the expression patterns of several cytokine mediators. Marcinkiewicz et al. [33] demonstrated that the increasing NO levels enhance the release of TNF-α and reactive oxygen species. This effect may be due to both an increased generation of superoxide anion, and the preferential formation of peroxynitrite, which can be formed by direct reaction between NO and the superoxide radical, and have powerful cytotoxic properties [34]. The decomposition product of peroxynitrite and the hydroxy radical is believed to be the most toxic oxygen molecule in vivo [35]. TNF-α and NO were investigated in the current study to confirm the possibility that CK might be an immunostimulator. CK was found to elicit NO and TNF-α production (Figs. 1 and 2). This result supports the possibility that NO and TNF-α induction of CK may contribute in vivo to the immunomodulating activities of CK. Because CK is known to be nontoxic, it will be assessed clinically. Biological response modifiers are widely used in cancer immunochemotherapy to potentiate therapeutic efficacy or to alleviate toxicity of cytotoxic anticancer agents. It is interesting that CK contains an immunostimulating compound and has been used as a herbal drug for a long period of time. The potent antitumor activity of CK is remarkable and, hopefully, it can be developed into an agent for cancer therapy. Further studies on the CK will be needed to prove its clinical usefulness in cancer therapy and in effectiveness in other diseases. In order to investigate the overall antitumor effect of CK, a study on the in vivo induction of gene expression and the production of immunomodulating cytokines in mice is in underway (manuscript in preparation). The exact mechanism underlying CK-induced NO and TNF-α production and release remains to be elucidated.

In summary, CK stimulates macrophage-derived NO and TNF-α production and is able to upregulate iNOS and TNF-α expression through NF-κB transactivation in murine macrophages. These actions might be source of its antitumor activity.

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