February 2008

Master's Thesis

Glycoprotein Isolated from *Dioscorea japonica* Activates Mouse Macrophages

Graduate School of Chosun University

Department of Bio New Drug Development

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Advisor: Prof. Cheol-Hee Choi

Thesis submitted for the degree of Master of Science

November 2007

Graduate School of Chosun University

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ABBREVIATIONS

BSA	Bovine serum albumin
DJ	Glycoprotein from *Dioscorea japonica*
DMEM	Dulbecco′s modified eagle′s medium
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
NO	Nitrite oxide
PKA	Protein kinase A (or cAMP-dependent protein kinase (cAPK)
PKC	Protein kinase C
PMSF	Phenylmethanesulphonylfluoride
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	Tris/boric acid EDTA buffer
TEMED	N, N, N′, N- Tetramethylethylenediamine

Abstract

Dioscorea japonica has been used not only in traditional Chinese medicine, but also in modern medicine to treat for uncontrollable urination, diabetes and chronic diarrhea. In this study, the effect of glycoprotein isolated from *D. japonica* (DJ) on the expression of TNF-α, IL-1β, COX-2 and iNOS was assessed in RAW 264.7 cells. DJ alone induced the expression of TNF-α and COX-2 genes, but neither induced the NO production nor iNOS gene expression. IL-1β gene expression was induced only in a higher concentration (25 μg/ml) of DJ. Western blot, RT-PCR and ELISA revealed that DJ in combination with INF-γ showed a strongly synergistic induction on NOS and IL-1β gene expression. Electrophoretic mobility shift assay showed that DJ alone and in combination with IFN-γ could activate NF-κB/Rel in a slightly synergistic manner. In addition, DJ alone strongly induced p44/42^{mapk}, and SAPK/JNK phosphorylation but not p38^{mapk} phosphorylation. In contrast, DJ in combination with IFN-γ synergistically activated p38^{mapk}, but only slightly activated p44/42^{mapk} and SAPK/JNK. The specific p38 inhibitor SB203580 partly inhibited NO production and iNOS gene expression. These data indicate that DJ stimulates RAW 264.7 cells through increasing gene expression of TNF-α, IL-1β and COX-2, which can be regulated by p44/42^{mapk} and SAPK/JNK pathways, and that DJ in combination with IFN-γ induce strongly NO production and iNOS gene expression in a synergistic manner, which can be mediated by p38 ^{mapk}. It could be implicated that DJ could be used as an immune modulator.

Key words: *Dioscorea japonica;* Macrophages; TNF-α; IL-1β; COX-2; iNOS; IFN-γ; MAPKs

국문초록

Discorea japonica **에서 분리한 당단백질에 의한 생쥐 대식세포의 활성화에 관한 연구**

Discorea japonica 는 동양의학에서 조절되지 않는 배뇨, 당뇨 및 만성 설사 등에 전통적으로 사용되어 왔다. 본 연구에서 *D. japonica* 에서 분리한 당단백질 (DJ)의 TNF-α, IL-1β, COX-2 및 iNOS 발현에 미치는 영향을 생쥐 대식세포 Raw 264.7 세포에서 관찰하였다. DJ 은 단독으로 TNF-α 및 COX-2 유전자의 발현을 유도하였으나, NO 생성 및 iNOS 유전자발현은 유도하지 못했다. DJ 는 높은 농도(25 μg/ml)에서만 IL-1β 유전자의 발현을 유도하였다. Western blot, RT-PCR 및 ELISA 분석으로 DJ 와 INF-γ 의 병용투여가 iNOS 와 IL-1β 유전자 발현의 유도에 강한 상승효과를 보인다는 것을 밝혔다. Electrophoretic mobility shift assay 는 DJ 가 단독으로 NF-kB/Rel 을 활성화시켰고, INF-γ 와 동시 투여시 약간 상승시킨다는 것을 보여주었다. 뿐만 아니라, DJ 는 단독으로 p44/42 MAPK 및 SAPK/JNK 인산화를 강력하게 유도하였으나, p38 MAPK 는 유도시키지 않았다. 반대로, DJ 와 INF-γ 를 병용투여한 경우 p38 MAPK 을 상승적으로 활성화시켰으나, p44/42 MAPK 및 SAPK/JNK 은 단지 약간만 활성화시켰다. p38 의 특이 억제제인 SB203580 은 DJ 와 INF-γ 의 병용 투여에 의해 유도되는 NO 의 생성과 iNOS 유전자 발현을 부분적으로 억제하였다. 이상의 결과는 DJ 가 TNF-α, IL-1β, COX-2 유전자 발현을 증가시킴으로써 Raw 264.7 세포를 활성화시키며, 이러한 효과는 p44/42 MAPK 및 SAPK/JNK 경로에 의해 조절될 수 있고, DJ 와 INF-γ 의 병용투여는 NO 의 생성과 iNOS 유전자 발현을 강력하게 상승적으로 유도하며, 이러한 효과는 p38 MAPK 에 의해 매개될 수 있다는 것을 의미한다. 그러므로 DJ 는 면역조절제로 사용될 수 있을 것으로 생각된다.

INTRODUCTION

Dioscorea species are plants that have been used not only in traditional Chinese medicine, but also in modern medicine as a major source of steroid precursors. The previous studies have been revealed that plants of this genus possess immunomodulatory activity *in vitro* on the viability, cell-mediated cytotoxicity and interferon-γ (INF-γ) secretion of splenic lymphocytes [1]. Phytochemical investigations have demonstrated that steroid saponins are active principles of *Dioscorea* species [2]. Among these, *Dioscorea japonica*, widely distributed in East Asia, has long been used in ethnomedicine for the treatment of poor appetite, chronic diarrhea, asthma, dry coughs, frequent or uncontrollable urination, diabetes, and emotional instability [2]. Here, I attempted to study the effect of DJ on the innate immunity by observing the inflammatory cytokine expression in macrophage cells.

Macrophages play major roles in both innate and acquired immunity. They can be stimulated by cytokines, such as IFN-γ, or microbial components, such as LPS [3, 4]. For innate immunity, macrophages phagocytise and kill microbes, release inflammatory mediators such as NO by the action of iNOS. For acquired immunity, macrophages serve as antigen presenting cells and release cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-1, IL-6, and IL-12 to regulate the functions or development of helper T cells [5]. Activated macrophages inhibit the growth of a variety of tumor cells and foreign organisms, such as microorganisms [6].

It has been repored that NO is involved in the cytolytic function of macrophages. NO is a highly reactive free radical and plays as an important second messenger molecule in

many cell types. It regulates many physiological and pathophysiological processes as well as in the maintenance of neuronal communication, vascular regulation and immune system [7]. Stimulation of murine macrophages by various stimuli like LPS and INF-γ, induces the expression of iNOS, which results in high-level NO production from L-arginine and molecular oxygen [7]. NO, in turn, participates in the inflammatory response of macrophages such as major cytotoxic mediator and inhibit the growth of invading microorganisms and tumor cells [8, 9]. The NF-κB/Rel family of transcription factors is composed of pleiotropic regulators of many genes involved in immune and inflammatory responses, especially the iNOS expression [5, 8]. The promoter of the murine gene encoding iNOS contains two κB-binding sites, located at 55 and 971 base pairs (bp) upstream of the TATA box, respectively [6]. Protein binding to the κB site is necessary to confer inducibility by LPS [10]. In unstimulated cells, NF-κB resides in the cytoplasm as a dimmer of protein components known as Rel family members (p50, p65 etc.) which is bound to an inhibitor (IκB). Upon stimulation or activation, IκB is phosphorylated and released from the complex, after that the complex undergoes proteolytic degradation and the Rel proteins migrate to the nucleus and bind to the cognate sites in the promoter regions of the genes for many inflammatory cytokines, resulting in their transcription [10, 11].

Most cellular responses to extracellular stimuli are mediated by cascades of kinase and phosphatase. One of the most important kinase families in inflammatory cells is MAPKs, which are strongly conserved through evolution, suggesting their vital role in intracellular signaling. p38 and p44/42 MAPKs are serine threonine kinases that are located in the

cytoplasm until activates by dual phosphorylation on both Tyr and Thr residues. p38 ^{mapk} have the Thr-Gly-Tyr dual phosphorylation motif, and p44/42 ^{mapk} are phosphorylated at Thr-Glu-Tyr motif [12]. The activation of p38 ^{mapk} through various pathways has been demonstrated to be essential for IL-1β, IL-6, TNF-α, COX-2 and iNOS expression. Especially, p38 kinase is played a key role in LPS-induced signal transduction pathways leading to cytokine synthesis [13, 14]**.** The activation of p38 ^{mapk} was proved to involve in iNOS expression in TNF-α and IL-1-stimulated mouse astrocytes as well as in LPS-stimulated mouse macrophages [15, 16].

In this study, I investigated the active effect of glycoprotein DJ on the expression of inflammatory mediators (IL-1β, TNF-α, COX-2), which may be mediated by p44/42^{mapk}, and SAPK/JNK kinase phosphorylation in macrophages. I also assessed the synergistic activation of DJ and IFN-γ on the NO production as well as iNOS expression in RAW 264.7 cells, which is mediated by p38^{mapk}, but not p44/42^{mapk} or SAPK/JNK kinase phosphorylation. I considered if DJ could affect on some modulatory immune responses through the signaling pathways.

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. DJ

Glycoprotein was isolated from *D. japonica* and characterized by Dr. DC Lee, Department of Biotechnology, Chungbuk Provincial College of Science and Technology. The morlecular weight of DJ is around 23kDa. Pure water was used as a solvent for DJ. LPS from *Salmonella typhosa* and IFN-γ were purchased from Sigma (MO, USA). SB203580 was purchased from Calbiochem (CA, USA).

2.1.2. RT-PCR assay

Tri-zol reagent Invitrogen (CA, USA), chloroform and isopropanol, Merk (Darmstadt, Germany), 10X TBE, and 10X DNA dye, 5 mg/ml bromophenol blue, 50% glycerol, 100 mM Tris, 20 mM NaCl and 1 mM EDTA were purchased from Biorad (CA, USA).

2.1.3. Antibodies

Anti-iNOS/NOS II (125 kDa), β-actin antibody (45 kDa), p38 antibody and phospho-p38 MAP Kinase (Thr180/Tyr182) antibody (43 kDa), p44/42 kinase antibody and phospho-p44/42 MAPKinase (Thr202/Tyr204) antibody (44; 42 kDa), JNK and phospho-SAPK/JNK (Thr183/Tyr185) antibody (46/54 kDa). IL-1β Antibody (17, 31 kDa), and goat anti rabbit IgG-HRP antibody were obtained from Cell Signaling Biotechnology, Inc (MA, USA) and Santa Cruz Biotechnology, Inc (CA, USA).

2.1.4. SDS-PAGE and Western Blot

Materials	Sources
40% Acrylamide/Bis solution	Bio-Rad
Tris-HCl	Sigma
Glycine	Sigma
TEMED	Amresco
SDS	Sigma
Methanol	Burdick & Jackson
Difco™ skimmilk	Becton, Dickinson
Hybond™-ECL™ Nitrocellulose membrane	Amersham Biosciences
β- Mercaptoethanol	Sigma
NaCl	Amresco
Tween -20	Sigma
BSA	Sigma
Na_2HPO_4	Amresco
Whatman paper	Whatman
Enhanced chemilumenescent detection Kit	Pierce, Rockford
Films	Fujifilm
Developer and rapid fixer	Vivid

2.1.5. EMSA

Hypotonic buffer (A): 10 mM HEPES (pH 7.9), 10 mM KCl, 1mM DTT, 0.1 mM EDTA, 0.1 mM EGTA and 0.5 mM PMSF.

Hypertonic buffer (B): 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF.

Gel shift binding 5x buffer: 20% Glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 0.25 mg/ml poly(dl-dC)*poly(dl-dC).

10% NP40; **5% PAGE** (0.5x TBE, 40% polyacrylamide, 10% APS, 1‰ TEMED) and sephadex G50.

Lysis buffer: 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 20 mM NaF, 50 mM PMSF, 1 mM Na_3VO_4, 100 μg/ml leupeptin, and 10 μg/ml aprotinin.

2.1.6. Cell culture

RAW 264.7 cells (murine mouse macrophage line) were purchased from American Type Culture Collection (MD, USA). Cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. Cells were then cultured in the presence of 5% CO_2 at 37°C.

2.2. METHODS

2.2.1. NO measurement

Nitrite accumulation was used as an indicator of NO production in the medium as previously described [17]. RAW 246.7 macrophage cells were plated at 3.5 x 10^5 cells/ml in 96-well culture plates and stimulated with DJ (1, 10, 25, and 50 μg/ml) in the presence

or absence of IFN-γ for 24 h. The supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve. Nitrite production was measured by an optical density at 540 nm.

2.2.2. Determination of IL-1β and TNF-α levels

RAW 264.7 cells were plated at 3.5 x 10^5 cells/ml in 24 well culture plates and stimulated with DJ (1, 10, and 25 μg/ml) in the presence or absence of IFN-γ (10 ng/ml) for 24 h. Cells were harvested and added with 100 μl H_2O and subjected to three freeze/thaw cycles to liberate intracellular TNF-α and IL-1β. The IL-1β and TNF-α levels were determined by using ELISA kit DuoSet^r ELISA Development System, R&D Systems, (MN, USA) according to the manufacturer's instruction.

2.2.3. Determination of protein concentration

The Bio-Rad protein assay kit was used for the quantification of protein. The standard curve was set up with BSA dilution in water as final concentration of: 0, 1.25, 2.5, 3.5, 5, and 10 μg/μl. The absorbance was measured at 690 nm.

2.2.4. SDS-PAGE and Western blot

At various time points after indicated treatment, RAW 264.7 cells were collected and washed with 1X PBS. Cells were lysed for 30 min in lysis buffer. Cell debris was removed by centrifugation (13,000 rpm, 15 min, 4^{o}C). Samples were boiled for 10 min. The boiled samples were loaded onto a 10% SDS-PAGE gel, and electrophoresed at 100 V for one hour. Protein was transferred into nitrocellulose membrane and reacted with various primary antibodies against different proteins. All immunoblots were stained with

horseradish peroxidase-linked secondary antibodies and visualized by enhanced chemiluminescence reagent.

2.2.5. RT-PCR assay

Total cellular RNA was isolated from RAW 264.7 cells using Trizol according to manufacture's protocol. The complimentary DNA strand (cDNA) was synthesized using Superscript II reverse transcriptase enzyme. Reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for iNOS, TNF-α, IL-1β, COX-2 and β-actin. Sequences of forward and reverse primers are: ***iNOS***: 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3', 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3'. **TNF-α**: 5'-CCT GTA GCC CAC GTC GTA GC-3', 5'-TTG ACC TCA GCG CTG AGT TG-3'. ***IL-1β***: 5'-TGC AGA GTT CCC CAA CTG GTA CAT C-3', 5'-GTG CTG CCT AAT GTC CCC TTG AAT C-3'. ***COX-2:*** 5'-TTT GTT GAG TCA TTC ACC AGA CAG AT-3', 5'-CAG TAT TGA GGA GAA CAG ATG GGA TT-3'. ***β-actin***: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'.

Equal amounts of RNA were reverse-transcribed into cDNA using oligo(dT15) primers. PCR was performed with cDNA and each primer. Samples were heated to 94^{o}C for 5 min and denaturated at 94^{o}C for 1 min, annealing of each primer during 1 min as follows: iNOS, TNF-α and IL-1β: 57^{o}C, COX-2: 55^{o}C, and β-actin: 53^{o}C, polymerization at 72^{o}C for 1 min and 25 cycles of each. At the last cycle, all PCR products were subjected to final extension at 72^{o}C for 7 min. The amplified products were mixed with 10 X DNA dye and separated by DNA electrophoresis on 1% agarose gel containing 0.2% ethidium bromide.

The iNOS, TNF-α, IL-1β, COX-2, and β-actin primers produce amplified products at 311 bp, 374 bp, 387 bp, 371 bp, and 349 bp, respectively.

2.2.6. EMSA

RAW 264.7 cells were grown at 5 x 10^5 cells/ml in petri-dish and then treated with DJ for 2 h in the presence or absence of IFN-γ. Nuclear extracts were prepared as earlier described [18]. Briefly, cells was washed with PBS, then resuspended and lysed by 400 µl of hypotonic buffer (A) and were allowed to swell on ice for 15 min. 25 µl of 10 % NP-40 was added then centrifugated at 12 000*g* for 1 min after vigorous vortexing for 10 s. The supernatant was removed and the nuclear pellet was extracted with 50 µl of hypertonic buffer (B) by shaking at 4^o C for 15 min. The extract was centrifuged at 12 000 *g* for 1 min. The supernatant was fozen at -80^o C for further use. The double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP. Nuclear extracts (5 µg) was incubated with in the gel shift binding 5 X buffer for 10 min and then added [^{32}P]-labeled DNA probe in each reaction according to the manufacture's protocol (Gel shift assay system, Promega Kit, USA). DNA binding NF-κB probe was separated from free probe using a 5% polyacrylamide gel in 0.5 X TBE buffer. Gel was dried and subjected to autoradioghraphy.

2.2.7. Statistic analysis

The mean $\pm$ SD was determined for each treatment group in a given experiment. Treatment groups were compared to the vehicle control using the Dunnett's two-tailed t-test [19].

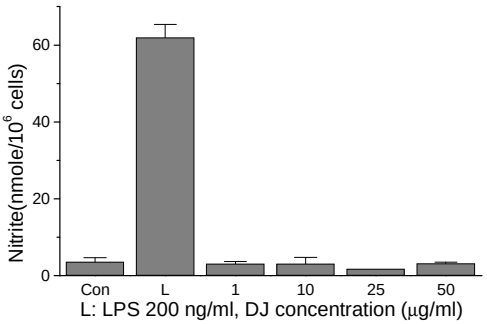
RESULTS

Part I: Macrophage activation by DJ

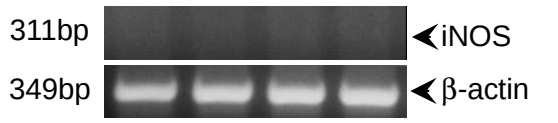
3.1.1. Effect of DJ on nitrite production and iNOS expression

The treatment of RAW 264.7 cells with DJ alone did neither induced NO production (Fig. 1A) nor iNOS gene expression (Fig. 1B and C). The similar results were also observed in peritoneal macrophages (data not shown). In addition, the morphology of RAW 264.7 macrophage cells was not changed after treatment of DJ (Fig. 1D). LPS is used as a positive control for reaction. Control β-actin was constitutively expressed and was not affected by DJ treatment. These results indicated that DJ alone could not activate mouse macrophages to induce iNOS gene expression.

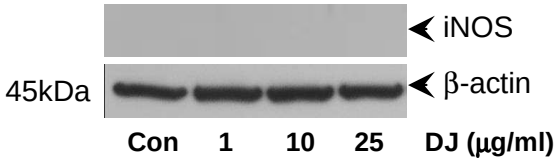
A


Nitrite(nmole/10^6 cells)
0
20
40
60
Con
L
1
10
25
50
L: LPS 200 ng/ml, DJ concentration (μg/ml)

B


311bp
iNOS
349bp
β-actin

C


iNOS
45kDa
β-actin
Con
1
10
25
DJ (μg/ml)

D

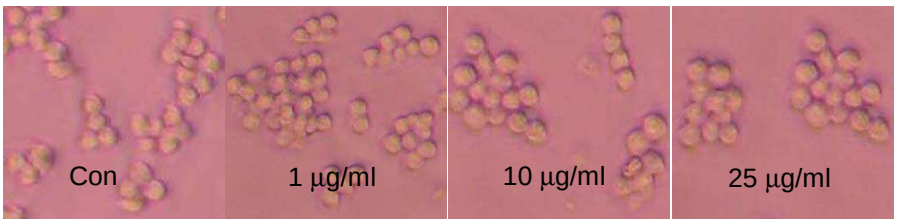
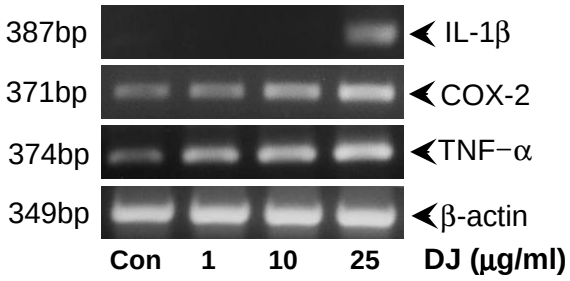

Con
1 μg/ml
10 μg/ml
25 μg/ml

Fig. 1. Effect of DJ on NO production and iNOS gene expression in RAW 264.7 cells. Cells were cultured at $3x10^5$ cells/ml and then treated with DJ (1, 10, and 25 μg/ml) for 24 h (**A**), 8 h (**B**) and 16 h (**C** and **D**). The culture supernatants were analyzed for NO production (**A**). iNOS and β-actin mRNA expression was analysed using RT-PCR assay, producing 311 bp and 349 bp respectively (**B**). Cell lysates were subjected to immunoblotting for protein productions of iNOS and β-actin (**C**). The morphology of RAW 264.7 cells in the treatment with DJ (Microscope lens LCAch.20X/0.40 php Olympus CKX41) (**D**). Each value shows the mean ± S.D. of triplicate determinations. One representative of three experiments is shown.

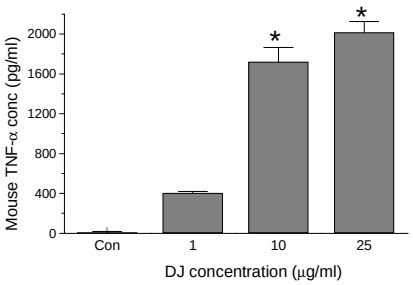
3.1.2. Effect of DJ on gene expression of some cytokines

In RT-PCR assay, DJ itself could stimulate macrophage cells to induce TNF-α and COX-2 gene expression in a concentration-dependent manner. At the higher concentration (25 μg/ml), DJ could induce IL-1β gene expression (Fig. 2A). The activation of IL-1β gene was the same pattern as iNOS expression. In ELISA, TNF-α and IL-1β were also obviously produced in a concentration-dependent manner (Fig. 2B and C). The same results were observed in peritoneal macrophages (the data not shown).

A


387bp
IL-1β
371bp
COX-2
374bp
TNF−α
349bp
β-actin
Con
1
10
25
DJ (μg/ml)

B


Mouse TNF−α conc (pg/ml)
2000
1600
1200
800
400
0
*
*
Con
1
10
25
DJ concentration (μg/ml)

C

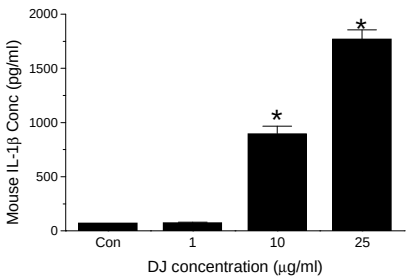

Mouse IL-1β Conc (pg/ml)
2000
1500
1000
500
0
*
*
Con
1
10
25
DJ concentration (μg/ml)

Fig. 2. Effect of DJ on gene expression of some cytokines in RAW 264.7 cells. Cells were maintained at 3 x 10^5 cells/ml and then incubated with DJ (1, 10, and 25 μg/ml) for 8 h (**A**) and for 24 h (**B** and **C**). IL-1β, COX-2, TNF-α, and β-actin mRNA expression was analysed using RT-PCR, producing 387 bp, 371 bp, 374 bp and 349 bp, respectively (**A**). The supernatants containing TNF-α from culture media and RAW 264.7 cells were harvested and subjected to mearure TNF-α (**B**) and IL-1β (**C**) by ELISA kit. One representative of three experiments is shown.

3.1.3. The activation of NF-κB/Rel by DJ

NF-κB/Rel was activated by DJ in a time-dependent manner (Fig. 3A). The DNA binding activity was determined 30 min after treatment. This binding activity was strong around 1 to 2 hours and then reduced. In addition, DJ activated NF-κB/Rel in a concentration-dependent manner. Both AP-1 and Oct had moderate basal binding activity and were not influenced by the DJ treatment (Fig. 3C).

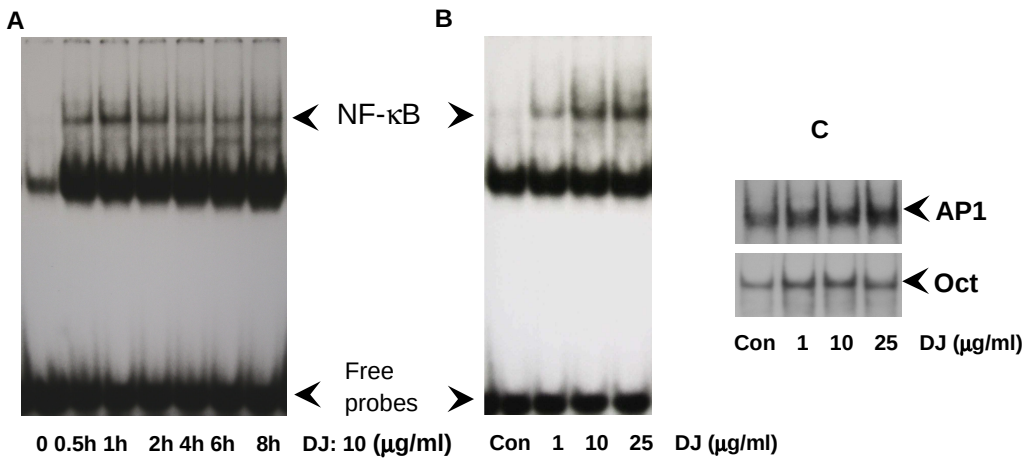


Fig. 3. The activation of NF-κB/Rel by DJ in RAW 264.7 cells. Cells were maintained at (5 x 10^5) cells/ ml and then treated with only DJ (10 μg/ml) as a function of time (**A**) and treated in each concentration (1, 10, and 25 μg/ml) for 2 h (**B** and **C**). Nuclear extracts were isolated and analyzed for the determination of NF-κB activity (**A** and **B**), AP1, and Oct (**C**). One representative of two experiments is shown.

3.1.4. The activation of p38, p44/42 and JNK by DJ

MAPKs is known to be activated by phosphorylation at threonine and tyrosine residues. Accordingly, when immunoblot analysis using p38, p44/42, and SAPK/JNK antibodies against phosphorylated tyrosine was performed, phosphorylation of p38 was slightly induced by DJ alone, whereas the phosphorylation of p44/42 MAPKs and especially of SAPK/JNK was markedly induced (Fig. 4).

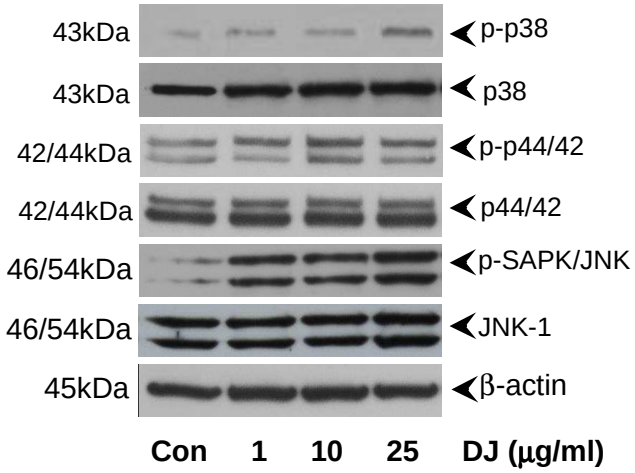


Fig. 4. Effect of DJ on the phophorylation of p38, p44/42, and SAPK/JNK in RAW 264.7 cells. Cells (3.5 x 10^5 cells/ml) were incubated with DJ (1, 10, and 25 μg/ml) for 2 h. Cell lysates were subjected to immunoblotting for p38, p44/42 and SAPK/JNK protein levels as well as phosphorylations. In the condition of serum starvation, the expression of these are the same, (data not shown). One representative of two experiments is shown.

Part II: Synergistic macrophage activation by DJ and IFN-γ

3.2.1. The synergistic effect of DJ and IFN-γ on NO production and iNOS gene expression

It was reported that IFN-γ interacts synergistically with LPS to induce transcription of iNOS [23, 24]. I tried to assess the effect of DJ (1, 10, and 25 μg/ml) in the presence of IFN-γ (10 ng/ml) in RAW 264.7 cells. There was increased markedly in the NO production (Fig. 5A). The similar results were also observed in peritoneal macrophages (the data not shown). In the presence of IFN-γ, DJ stimulated macrophages to induce NOS gene expression in a concentration-dependent manner (Fig. 5B and C). Control β-actin was constitutively expressed and was not affected by the DJ and IFN-γ treatment. These results reflected that the increseased production of NO was mediated by the expression of iNOS gene. This seems the same the behavior of LPS interacts synergistically with IFN-γ to induce NOS gene expression as reported before. Morphology of RAW 264.7 cells was changed after treatment of DJ in the presence of IFN-γ (Fig. 6). These were also reflected by iNOS immunostaining (Fig. 7). Immunoreactivity of iNOS was localized along the margin of the cytoplasm of control sample. After incubation with DJ in the presence of IFN-γ, the expression of iNOS was strongly increased in the cytoplasm of activated RAW 264.7 cells. This indicated that the increase of NO was derived from the induction NOS production. These data showed that DJ synergistic strongly with IFN-γ in the activation of macrophages to induced iNOS gene expression in a concentration-dependent manner.

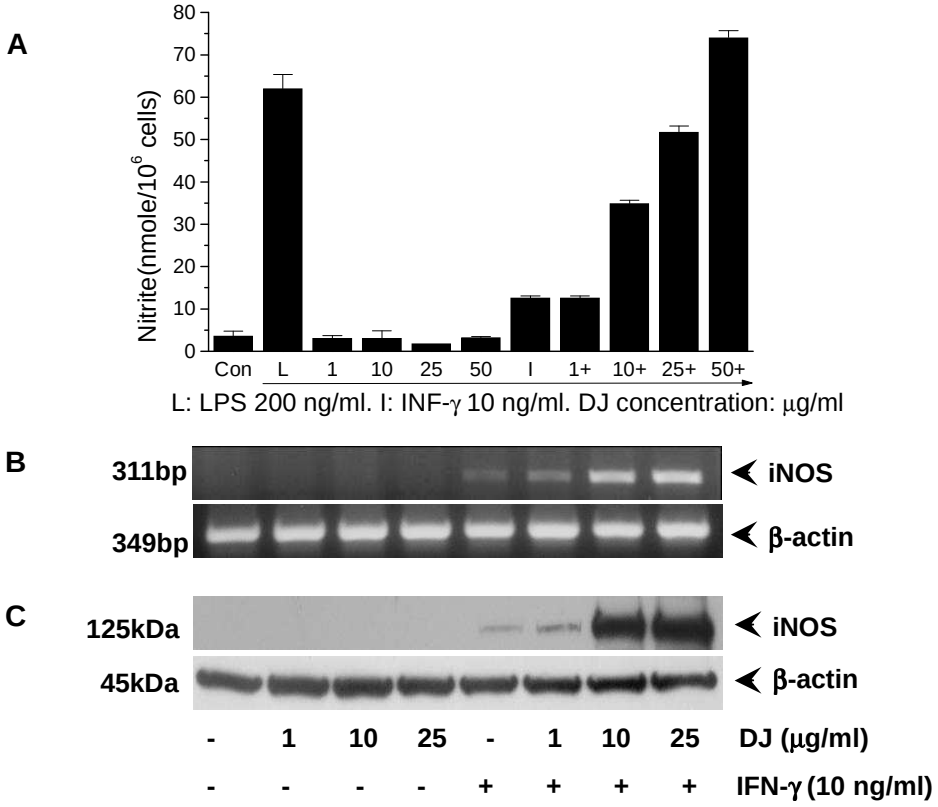


Fig. 5. The synergistic effect of DJ and IFN-γ on the NO production and iNOS gene expression in RAW 264.7 cells. Cells were maintained at 3 x 10^5 cells/ml and then treated with DJ (1, 10, and 25 μg/ml) in the absence and presence of IFN-γ (10 ng/ml) for 24 h (**A**), 8 h (**B**) and for 16 h (**C**). The culture supernatants were analyzed for NO production (**A**). LPS (200 ng/ml) was used as the positive control. iNOS and β-actin mRNA expression was analysed using RT-PCR assay, producing 311 bp and 349 bp respectively (**B**). Cells lysates were then subjected to immunoblotting for iNOS and β-actin protein production (**C**). Each value shows the mean ± S.D. of triplicate determinations. One representative of three experiments is shown.

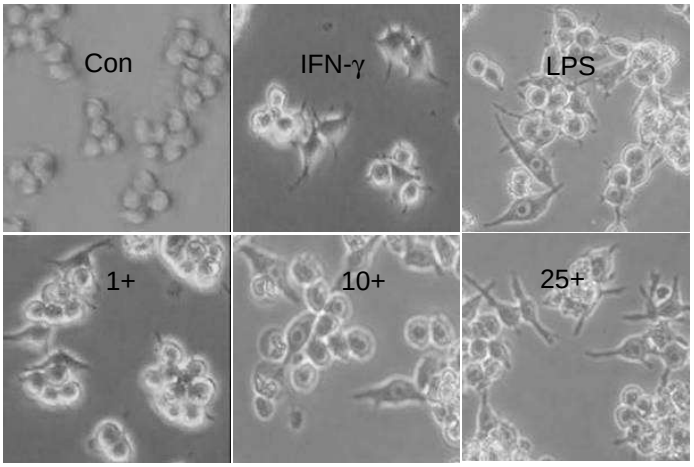


Fig. 6. The synergistic effect of DJ and IFN-γ on the morphology of RAW 264.7 cells. Cells were treated DJ (1, 10 and 25 μg/ml) in the presence of IFN-γ (10 ng/ml). Microscope lens LCAch.20X/0.40 php Olympus CKX41 showed the changes in morphology of RAW 264.7 cells in a concentration-dependent manner.

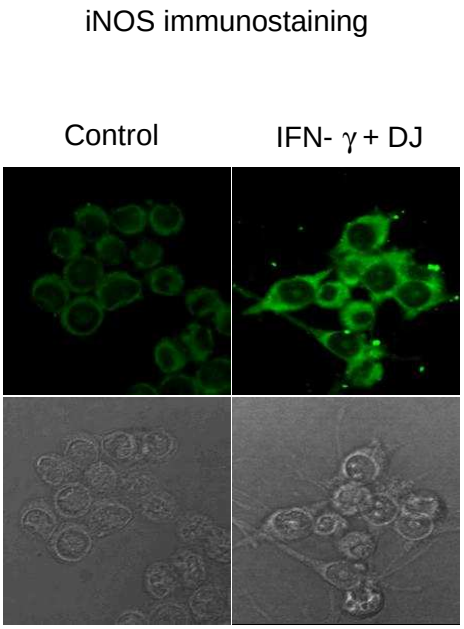
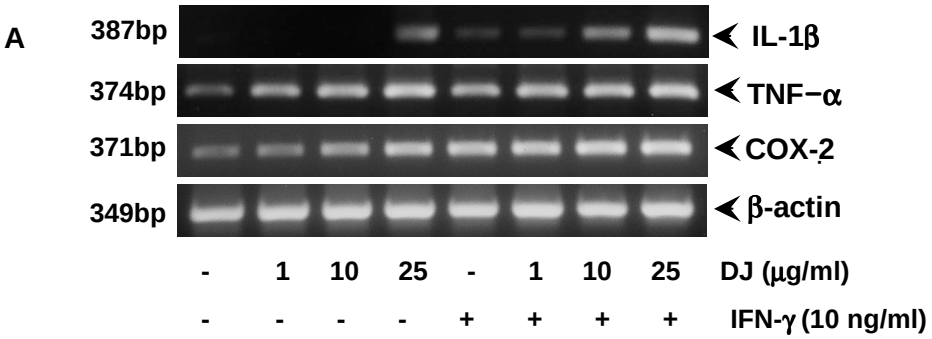
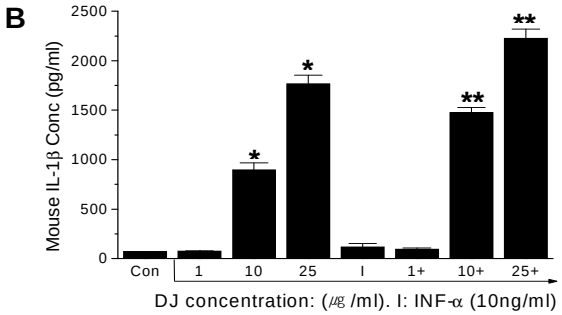


Fig. 7. Immunohistochemical staining of iNOS in RAW 264.7 cells. Cells (5 x 10^5 cells/ml) were incubated with DJ (25 μg/ml) for 24 h on cover slide in 12 well plates. Cells were subjected to immunohistochemical staining using an antibody specific for murine iNOS.

3.2.2. The synergistic effect of DJ and IFN-γon gene expression of some cytokines

It was reported that iNOS gene expression is involved in anti-tumor activity and inflammatory mediate processing in macrophages [14, 22]. I used RT-PCR and ELISA to assess the effect of DJ in the presence of IFN-γ on gene expression of some cytokines in RAW 264.7 cells. DJ was strongly synergistic with IFN-γ to induce IL-1β mRNA expression in a concentration-dependent manner (Fig. 8A). This was also notabled in the production of IL-1β measured by ELISA (Fig. 8B). At 25 μg/ml, the synergistic interaction of DJ and IFN-γ to produce IL-1β is very strong. The similar results were observed in peritoneal macrophage cells (data not shown). While DJ was just slightly synergistic with IFN-γ to induce TNF-α and COX-2 gene expression (Fig. 8A and C).


A
387bp
374bp
371bp
349bp
IL-1β
TNF−α
COX-2
β-actin
- 1 10 25 - 1 10 25 DJ (μg/ml)
- - - - + + + + IFN-γ (10 ng/ml)


B
Mouse IL-1β Conc (pg/ml)
2500
2000
1500
1000
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0
Con 1 10 25 I 1+ 10+ 25+
DJ concentration: (μg /ml). I: INF-α (10ng/ml)

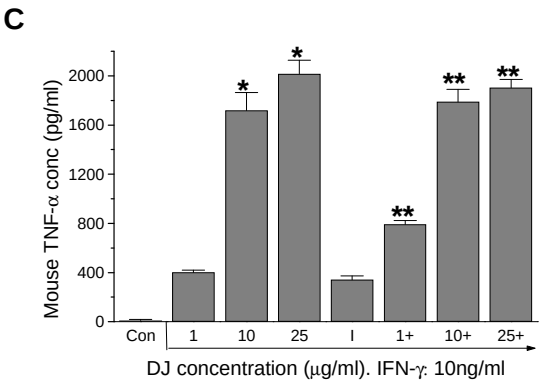
C
Mouse TNF-α conc (pg/ml)
2000
1600
1200
800
400
0
Con 1 10 25 I 1+ 10+ 25+
DJ concentration (μg/ml). IFN-γ: 10ng/ml

Fig. 8. The synergistic effect of DJ and IFN-γ on gene expression of some cytokines in RAW 264.7 cells. Cells were maintained at 3.5 x 10^5 cells/ml and then treated with DJ (1, 10, and 25 μg/ml) in the presence or absence of IFN-γ (10 ng/ml) for 8 h (**A**) and 24 h (**B** and **C**). mRNA expression was analyzed for IL-1β (387 bp), COX-2 (371 bp), TNF-α (374 bp), and β-actin (349 bp) using RT-PCR assay (**A**). Cells were harvested and subjected to mearure IL-1β (**B**) and TNF-α contained in the supernatants of the culture media were also measured by ELISA kit (**C**). One representative of three experiments is shown.

3.2.3. The synergistic activation of DJ and IFN-γon NF-kB/Rel

The nuclear translocation and DNA binding of NF-κB/Rel are known to be preceded by the phosphorylation and degradation of IκBα. The DNA binding of NF-κB/Rel is known to be preceded by the nuclear translocation of NF-κB/Rel. To investigate whether NF-κB/Rel was activated by DJ and IFN-γ, EMSA was performed. DJ and IFN-γ (10 ng/ml) was slightly synergistic to activate NF-κB/Rel in a time-dependent manner (Fig. 9**A**). However, when DJ (1, 10, and 25 μg/ml) was treated on RAW 264.7 cells in the presence or absence of IFN-γ for 2 h, DJ was not significantly synergistic with IFN-γ in the activation NF-κB/Rel in a concentration-dependent maner (Fig. 9**B)**. The effect of DJ in the presence of IFN-γ on the AP-1 and Oct whose binding motifs are in the promoter of iNOS gene was also assessed. both of AP-1 and Oct had moderate basal binding activity and was not influenced by co-treatment of DJ and IFN-γ (Fig. 9C).

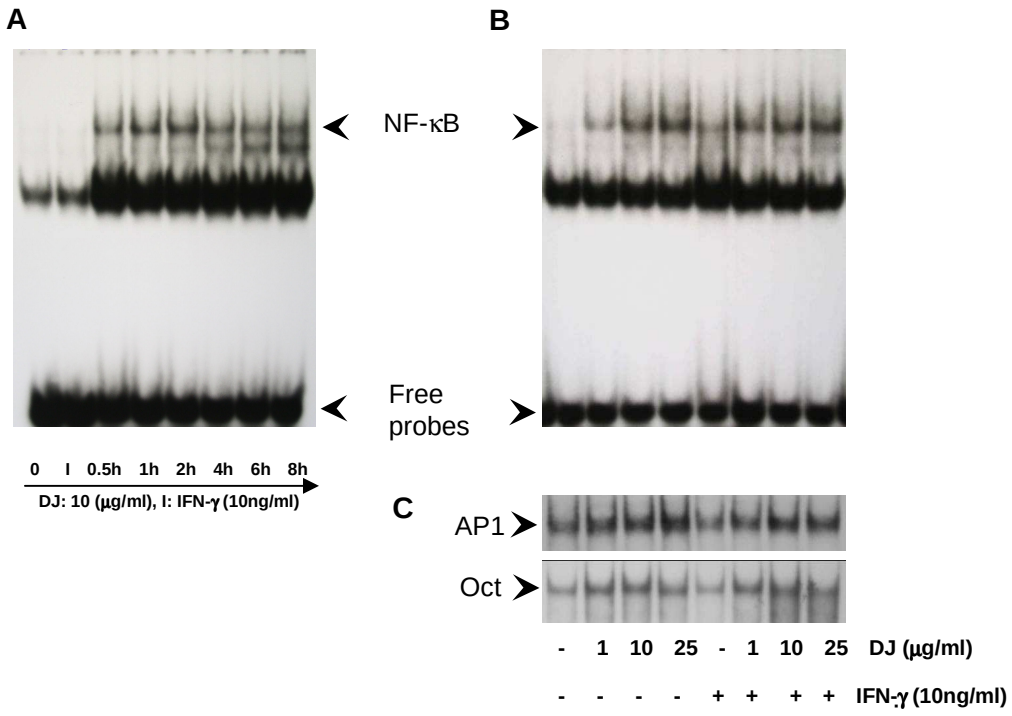

Fig. 9. The synergistic activation of DJ and IFN-γ on NF-κB/Rel in RAW 264.7 cells. Cells were maintained at 5 x 10^5 cells/ml and then treated with DJ (10 μg/ml) in the presence of IFN-γ (10 ng/ml) in the indicated time (**A**), and treated with a dose (1, 10, and 25 μg/ml) in the presence or absence of IFN-γ for 2 h (**B** and **C**). Nuclear extracts were isolated and analyzed for the activity of NF-κB (**B**), AP1, and Oct (**C**).

3.2.4. The synergistic effects of DJ and IFN-γ on the phosphorylation of p38^{mapk}, p44/42 and SAPK/JNK

Co-treatment of DJ and IFN-γ strongly induced p38 phosphorylation in a concentration-dependent manner, but slightly induced p44/42^{mapk} and SAPK/JNK phosphorylation in RAW 264.7 cells (Fig. 10). These data suggested that DJ might be synergistic with IFN-γ in the activation of p38 is the same on iNOS gene expression partern.

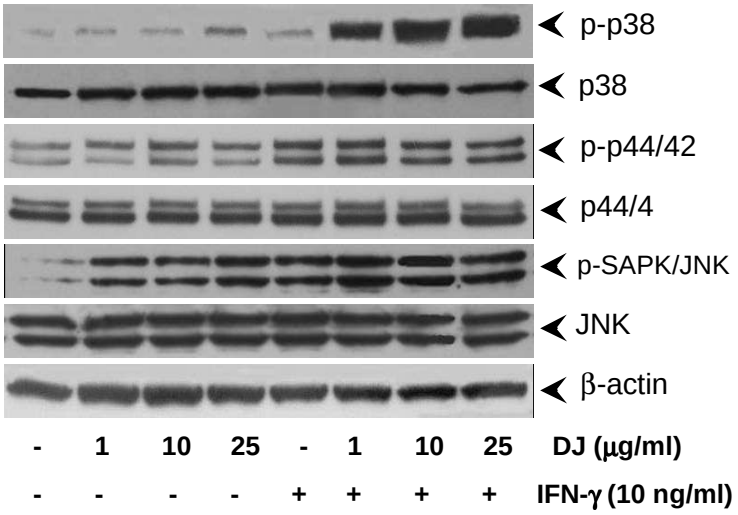

Fig. 10. The synergistic effects of DJ and IFN-γ on the phosphorylation of $p38^{mapk}$, p44/42 and SAPK/JNK in RAW 264.7 cells. Cells (3.5×10^5 cells/ml) were treated with DJ (1, 10, and 25 μg/ml) in the presence or absence of IFN-γ (10 ng/ml) for 20 min. Cell lysates were subjected to immunoblotting for p38, p44/42, and JNK proteins as well as phosphorylated forms. In the serum starvation condition, their expressions were the same (the data not shown). One of three representative experiments is shown.

3.2.5. The effect of p38 inhibitor SB203580 on iNOS gene expression in RAW 264.7 cells

The finding that DJ was strongly synergistic with IFN-γ to activate p38^{mapk} suggested that p38 kinase pathway would be involved in nitrate generation induced by co-treatment of DJ and IFN-γ. Accordingly, to verify this finding, we pretreated RAW 264.7 cells with SB203580, the specific inhibitor for p38^{mapk} as a bicyclic imidazole compound [25], for 30 min before the co-treatment of DJ and IFN-γ, and monitored NO production. Both NO production and iNOS expression were partly inhibited by SB203580 (Fig. 11). These results indicate that the p38 kinase pathway is important in the regulation of iNOS expression in RAW 264.7 cells activated by the co-treatment of DJ and IFN-γ.

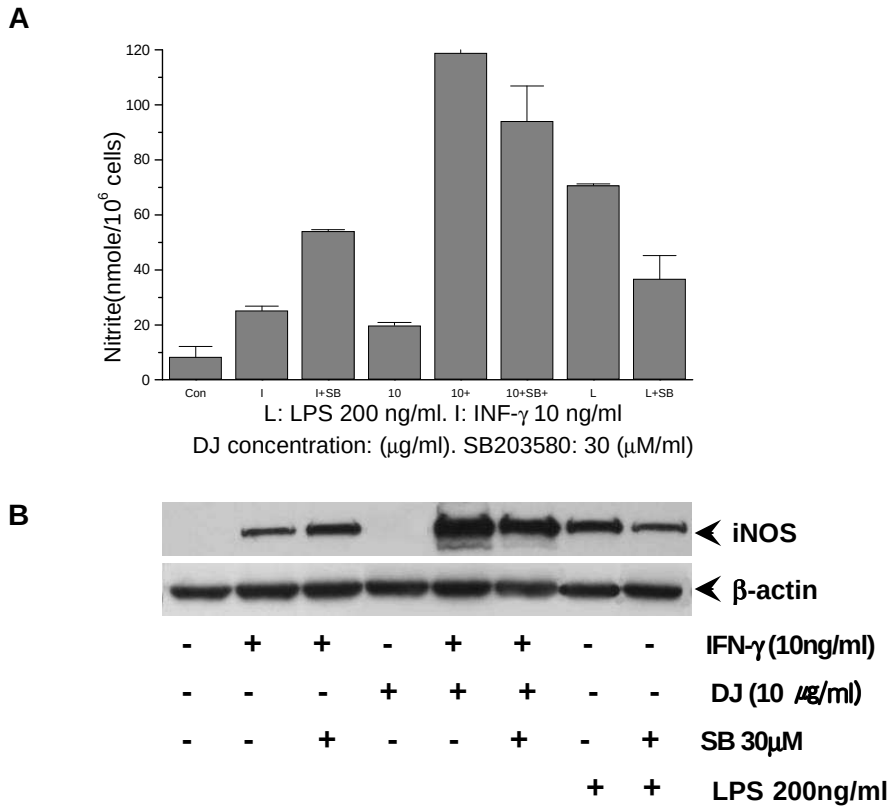

Fig. 11. The effect of p38 inhibitor SB203580 on iNOS gene expression in RAW 264.7 cells. Cells were pretreated with SB203580 (30 μM) for 30 min and then treated with DJ (10 μg/ml) and IFN-γ (10 ng/ml) for 24 h (**A**) and 16 h (**B**). The culture supernatants were analyzed for NO production (**A**). LPS (200 ng/ml) was used as the positive control. Cell lysates were then subjected to immunoblotting for the protein production (**B**). One of two representative experiments is shown.

DISCUSSION

The treatment of DJ alone induced significantly TNF-α and COX-2. However, DJ could not induce NO production and iNOS expression (Fig. 1). IL-1β induction was required higher concentration (25 μg/ml) of DJ (Fig. 2**A** and **C**). Moreover, DJ itself can activate NF-κB/Rel in the RAW 264.7 cells (Fig. 3). Normally, NO production is a precursor for the production of cytokines. The activated-macrophages produce NO to response to pro-inflammatory stimuli and then the resulting excess amounts of NO lead to increased production of inflammatory cytokines [13, 14]. However, according to the results, DJ directly induced some cytokines without NO production in the activated-RAW 264.7 cells.

The MAPK pathways have the key roles in the regulation of cell proliferation, differentiation, development, inflammation, survival, and migration [26]. DJ alone affected on the phosphorylation of p44/42 and SAPK/JNK in a concentration-dependent manner in RAW 264.7 cells while it was just slightly affected on the phosphorylation of $p38^{mapk}$ (Fig. 4). It has been reported that some components for example polysaccharide (PCSC) stimulate RAW 264.7 murine macrophages to activate NF-κB/Rel family [13]. In this study, DJ-stimulated RAW 264.7 cells significantly induces TNF-α, COX-2 and activated NF-κB/Rel transcription factors, which were possibly mediated by $p44/42^{mapk}$ and SAPK/JNK activation, but not by $p38^{mapk}$ activation.

The synergistic activation of DJ and IFN-γ in RAW 264.7 cells was remarked through the iNOS and some cytokines gene expression. Especially, p38 kinase pathway was involved in the regulation of iNOS expression in RAW 264.7 cells activated by the co-treatment of DJ and IFN-γ.

Although DJ was slightly synergistic with IFN-γ to activate NF-κB/Rel (Fig. 9), NO production and iNOS gene expression were strongly increased (Fig. 5, 6 and 7) in a concentration-dependent manner. DJ was slightly synergistic with IFN-γ to induce TNF-α and COX-2 expression, but strongly synergistic with IFN-γ to induce IL-1β gene expression (Fig. 8). It has been examined the role of p44/42 and $p38^{mapk}$ in the expression of NO and iNOS expression by LPS in primary glial cells [31]. It has also reported recently that $p38^{mapk}$ augmented iNOS induction by LPS, but that $p44/42^{mapk}$ was not involved [20]. Moreover, MAPKs are important regulators of iNOS-$NO^{\cdot}$ expression by co-treatment of IFN-γ and LPS [23]. The p38 kinase is an important mediator of stress-induced gene expression [12]. And p38 kinase activation is required to transduce signals leading to NF-κB/Rel activation and NO production in PCSC-stimulated murine macrophages [13]. In this study, only DJ treatment cannot activate macrophages to induce NO production as well as iNOS gene expression. Moreover, DJ itself seems to be no effect on $p38^{mapk}$ phosphorylation (Fig. 4). The phosphorylation of $p38^{mapk}$, NO production and iNOS gene expression in RAW 264.7 cells were derived from the synergistic activity by co-treatment of DJ and IFN-γ. This is matched with many reports that $p38^{mapk}$ phosphorylation involved in the signaling pathway stimulating NF-κB/Rel, which is major activating promoter of iNOS and some cytokines gene expression.

However, the involvement of p38 kinase and iNOS expression is controversial. It has been reported that selective p38 kinase inhibitor SB203580 has no effect on iNOS expression in LPS-induced RAW 264.7 cells [33]. And SB203580 has no effect on IFN-γ/TNF-α-induced iNOS expression in mouse macrophages [34]. In contract, using

dominant negative p38 kinase mutants, the involvement of p38 kinase activation in iNOS induction by IL-1β was proved in rat mesangial cells [35]. It was also demonstrated that p38 kinase activation is involved in iNOS expression in TNF-α and IL-1β-stimulated mouse astrocytes, as well as in LPS-stimulated mouse macrophages [9]. In this study, NO production, the expression of iNOS and IL-1β genes in RAW 264.7 cells was mediated by $p38^{mapk}$ through the activation of NF-κB/Rel. These findings indicate that p38 kinase pathway is an important mediator of iNOS production by the co-treatment of DJ and IFN-γ. In the presence of IFN-γ, DJ strongly induced the p38 activation in RAW 264.7 cells (Fig. 11). Especially, SB203580 blocked partly the NO production and iNOS expression by co-treatment of DJ and IFN-γ in RAW 264.7 cells (Fig. 12).

The induction of NOS by LPS in RAW 264.7 cells involves the binding of NF-κB heterodemers p50/c-Rel and p50/RelA to the NF-κB site at -85 to -75 in the iNOS promoter [8]. It has been reported that both LPS and DJ are large-size molecules and cannot penetrate into cells. In the case of LPS, the molecule binds to CD14 and activated signal transduction systems, such as PKC and PKA [27, 28]. Through a series of signaling pathways, IκB and inhibitor of NF-κB/Rel are phosphorylated and degraded, and NF-κB/Rel proteins are converted to an active form to activate the transcription of iNOS gene. The activation of NF-κB/Rel has an important regulatory role in the main immuno-regulatory genes that are involved in immune and inflammatory responses, such as some cytokines and iNOS gene [8, 29]. Especially the NF-κB/Rel activation in LPS-stimulated macrophages induces NOS gene expression and some cytokines were been known [28]. Expression of iNOS generally requires exposure of cells to immune or inflammatory

stimuli [17]. This phenomenon was demonstrated with mouse peritoneal macrophages treated with bacterial LPS, in which NO production was enhanced synergistically by the addition of IFN-γ [4, 30]. And NO is related to cytolysis function of macrophages against a variety of tumors, the increased synthesis of NO might interfere with the growth of tumors [31]. NO production is the first signaling of iNOS gene expression. The expression of iNOS is regulated largely at the transcriptional level has been formally demonstrated in mouse macrophages [7], where it has also been shown that the synergistic interaction between LPS and IFN-γ in the induction of iNOS is itself largely transcriptional [32]. Moreover, co-treatment of LPS and IFN-γ induction of iNOS is modulated by p44/42, SAPK/JNK, and $p38^{mapk}$ in mouse macrophages [23]. Since the macrophage activation by the co-treatment of DJ and IFN-γ was quite similar to that by the co-treatment of LPS and IFN-γ, I supposed that co-treatment of DJ and IFN-γ somehow followed the same signaling pathway as LPS and IFN-γ did in immune cells, however, there were some discrepancies observed between them.

In summary, these results suggested that DJ activates RAW 264.7 cells to produce some cytokines such as TNF-α, IL-1β, COX-2 through the activation of NF-κB/Rel transcription factors, which could be modulated by p44/42, SAPK/JNK but not $p38^{mapk}$. The production of NO and iNOS gene expression were assessed. Co-treatment of DJ and IFN-γ activated synergistically to induce NOS gene expression in RAW 264.7 cells, which could be mediated by $p38^{mapk}$ phosphorylation. Most of mechanisms behind this biological effect involve in the activation of NF-κB/Rel transcription factors. The main of this study showed a potentiality for anti-tumor activity by glycoprotein in macrophages.

CONCLUSION

DJ stimulates RAW 264.7 cells through increasing the expression of inflammatory mediators (IL-1β, TNF-α, COX-2), which could be regulated by $p44/42^{mapk}$ and SAPK/JNK pathways. The synergistic activation by co-treatment of DJ and IFN-γ in macrophages induced strongly NO production and iNOS gene expression, which could be mediated by $p38^{mapk}$ pathway.

This effect of DJ on macrophages could be used for studying the inflammatory mediator regulatory mechanism.

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Gwangju, Dec 2nd, 2007

Pham Thi Thu Huong

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