MIYABENOL A EXHIBITS ANTI-INFLAMMATORY ACTIVITY THROUGH DOWN-REGULATION OF LPS-INDUCED NF-κB AND JAK/STAT PATHWAYS IN RAW 264.7 MACROPHAGES

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We have previously reported that miyabenol A (a stilbene isolated from the roots of *Vitis thunbergii*) at concentrations ranged from 0.1 to 10 μM displayed potent anti-inflammatory activity to inhibit lipopolysaccaride (LPS)-stimulated nitric oxide (NO) production and inducible NO synthase (iNOS) expression in RAW 264.7 macrophages. Herein, we further investigated its mechanism of action focusing at the transcription level. Cells stimulated with LPS evoked a significant nuclear translocation of NF-κB, which was associated with cytosolic degradation of IκB. These activating phenomenon were abrogated in the presence of miyabenol A (3 and 10 μM). LPS also triggered AP-1 activation as revealed by increased c-Jun phosphorylation and c-Fos nuclear translocation (two major component of AP-1), but miyabenol A failed to affect this activation process. However, miyabenol A reduced LPS-activated phosphorylations of JAK-1/STAT-1. In summary, miyabenol A may inhibit LPS-mediated inflammatory response through down-regulation of both the NF-κB and JAK/STAT pathways to interrupt iNOS expression in RAW 264.7 macrophages.

Key words: *Vitis thunbergii*, Miyabenol A, LPS, iNOS, NF-κB, AP-1, JAK/STAT

Introduction

The roots of *Vitis thunbergii* Sieb. & Zucc. (or *Vitis ficifolia* Bge., Vitaceae) are traditionally used for the treatment of diarrhea, fracture and injury, jaundice, and hepatitis in Taiwan[1]. We have previously reported that miyabenol A, a stilbene isolated from the roots of *Vitis thunbergii*, inhibited lipopolysaccaride (LPS)-induced inducible nitric oxide synthase (iNOS) mRNA and protein expressions in RAW 264.7 macrophages[2]. Excepting physiological roles, NO participates in diverse pathogenesis such as

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inflammation and autoimmune diseases[3, 4]. Therefore, the inhibition of NO production by blocking iNOS expression may be a useful strategy for the treatment of various inflammatory disorders.

The expression of the iNOS gene in macrophages is regulated mainly at the transcriptional level[5]. The well-known transcription factor nuclear factor-κB (NF-κB) is a pivotal regulator of important immunoregulatory genes that are involved in immune and inflammatory responses, including iNOS[6]. NF-κB exists primarily in the cytoplasm as homo- or hetero-dimers complexed with inhibitory IκB proteins. Upon activation with signaling molecules such as LPS, IκB undergoes phosphorylation, ubiquitination, and proteasome-mediated degradation, and the NF-κB released from IκB is translocated into the nucleus, where it binds to the promoter of iNOS gene and activates transcription[7, 8]. Activator protein-1 (AP-1), another early transcriptional factor, is also involved in iNOS gene transcription either alone or by coupling with NF-κB[9]. Like NF-κB, AP-1 is consisted of homo- or hetero-dimers of the Jun family (c-Jun, JunB, and JunD) and Fos family (c-Fos, FosB, Fral, and Fra2) and can be activated by LPS[10]. In addition to NF-κB and AP-1, the Janus kinase-signal transducer and activator of transcription (Jak/STAT) pathways control a distinct, but interrelated, arm of the inflammatory response to microbial infection[11]. STATs are cytosolic proteins and are activated by tyrosine phosphorylation mediated by the interferon/ growth factor receptor-associated kinases, Jak and Tyk[12]. Activated STATs then form homo- or hetero-dimers through intermolecular SH2 phosphotyrosine interactions and are subsequently translocated into the nucleus in distinct binding complexes dictated by the nature of the stimulus and target promoters[13]. Because miyabenol A (0.1-10 μM) was found to suppress iNOS induction in LPS-stimulated RAW264.7 macrophages without any cytotoxic effect, this study was focusing at the transcriptional levels of iNOS to determine its action mechanisms.

Materials and methods

Chemicals and antibodies

Miyabenol A was isolated by Dr. Huang's laboratory and more detailed information for the HPLC analysis was described in previous work[14]. The purity for this compound determined by high-performance liquid chromatography with an UV detector (254 nm) was 99.8 %. Lipopolysaccharide (LPS) (Escherichia coli, serotype 055:B5) was purchased from Sigma Chemical (St. Louis, MO, USA). The antibodies for detecting the expression of IκB-α, NF-κB p65 and β-actin protein were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, 2145 Delaware Avenue, CA 95060, USA). The antibodies for total and phosphorylated STAT-1, c-Jun and c-Fos were obtained from Cell Signaling (Cell Signaling Biotechnology, Beverly, MA, U.S.A.). All chemical solvents were purchased from Merck KGaA (Darmstadt, Germany).

Cell Culture

The RAW 264.7 macrophage cell line (BCRC 60001) was obtained from the Bioresource Collection and Research Center of Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island, NY 14072, USA) supplemented with antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and

10% heat inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek 25115, Israel) and maintained at 37 °C in 5% CO_2 humidified air [15].

Preparation of total cell lysate for Western blot analysis

After pretreated with miyabenol A for 30 min, cells were stimulated with 1 μg/ml LPS for 30 min and performed protein extraction as described in the following. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenyl- methylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride and 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated with 20 min at 4 °C. After removal of cell debris by microcentrifugation the total protein was obtained. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instruction. Forty micrograms of cellular proteins from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation on 8% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, followed by incubation for 2 h with a primary antibody. Blots were washed four times with Tween 20/Tris-buffered saline (TTBS) and incubated with appropriate dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science) [16].

Preparation of nuclear extraction for translocation assay

Nuclear extracts of RAW264 macrophages before and after miyabenol A treatment were prepared using NE-PER nuclear and cytoplasmic extraction reagent (Pierce, 3747 N. Meridian Road, Rockford, IL 61105, USA) according to the manufacturer' s instruction supplemented with a set of protease inhibitors (Pierce). Thirty micrograms of nuclear protein extract was denatured in Laemmli buffer and separated using 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring, membrane was detected by antibodies against NF-κB p65 and c-Fos then detected by ECL (Amersham). Nucleolin (Chemicon International Inc.) served as a loading control for nuclear extract[16].

Statistical analysis

Data are expressed as mean±SE. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student's t-test for each paired experiment. A P value < 0.05 or 0.01 was considered as statistically significant difference.

Results

Effect of miyabenol A on LPS-induced IκB-α degradation and NF-κB nuclear translocation

In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor, IκB, which is phosphorylated upon LPS stimulation and then ubiquitinated and rapidly degraded via 26S proteosome to release NF-κB [17]. Here, we investigated whether miyabenol A inhibits the LPS-

stimulated degradation of IκB-α in RAW 264.7 macrophages by Western blotting with anti-IκB-α antibody. Figure 1 (upper) shows that LPS-induced IκB-α degradation was significantly blocked by 3 and 10 μM miyabenol A pretreatment. We also investigated whether miyabenol A prevents the translocations of the p65 subunit of NF-κB from the cytosol to the nucleus after their release from IκBs. It was found that treatment with miyabenol A attenuated p65 level in nuclear fractions by Western blotting in a concentration-dependent manner (Figure 1**,** lower); β-actin and nucleolin were used as internal controls, respectively. These findings indicate that miyabenol A may inhibit NF-κB activation by interfering with IκB-α degradation in LPS-induced RAW 264.7 cells.

Effect of miyabenol A on LPS-induced AP-1 activation

It has been reported that AP-1 regulates iNOS expression by binding to the iNOS promoter [18]. Thus, we tested the effect of miyabenol A on LPS-induced AP-1 activation. RAW264.7 cells were pretreated with miyabenol A for 30 min before exposure to LPS. The activation of c-Jun and c-Fos, two major components of AP-1, were individually assessed using phosphorylated c-Jun antibody immunoblotted with total cell lsyate and using control c-Fos antibody immunoblotted with nuclear protein extract. As shown in Figure 2, neither LPS-induced c-Jun phosphorylation nor c-Fos nuclear translocation was affected by miyabenol A, suggesting that miyabenol A suppressed LPS-induced iNOS expression was not attributable to AP-1 inhibition.

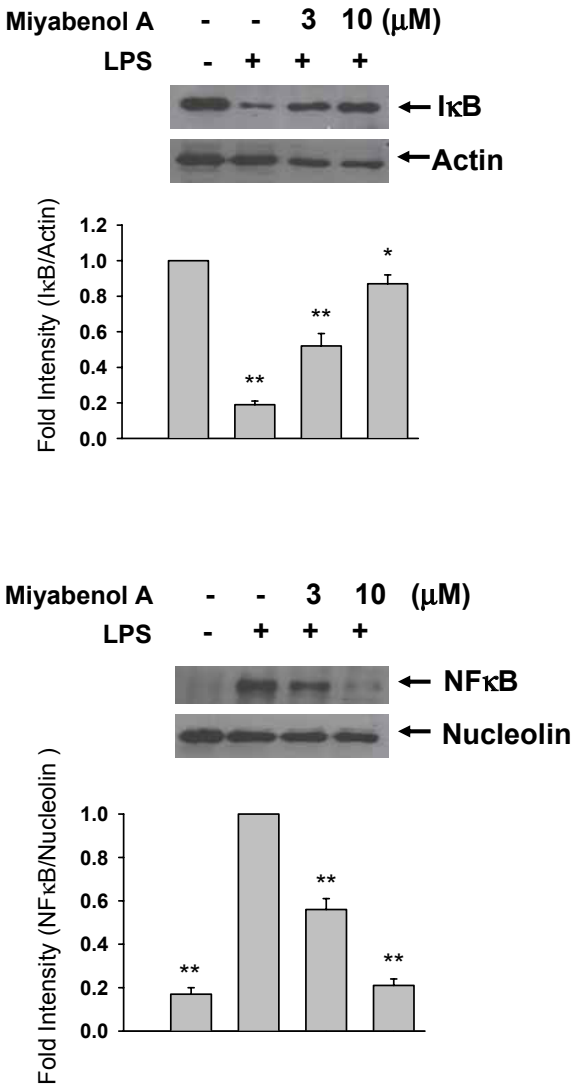

Fig. 1 Effects of miyabenol A on lipopolysaccharide (LPS)-stimulated IκB degradation and NF-κB nuclear translocation in RAW264.7 macrophages. Cells were treated with miyabenol A (3 and 10 μM) for 30 min, and then exposed to LPS (1 μg/ml) for 30 min. Cytosol and nuclear extracts were assessed by Western blotting and protein signals were detected with antibody against IκB-α, NF-κB p65, actin and nucleolin, respectively. Data reported are mean±SE of four independent experiments. * *P*<0.05 and ** *P* <0.01, indicate significance of difference as compared with control without LPS stimulation (upper) or samples receiving LPS alone (lower).

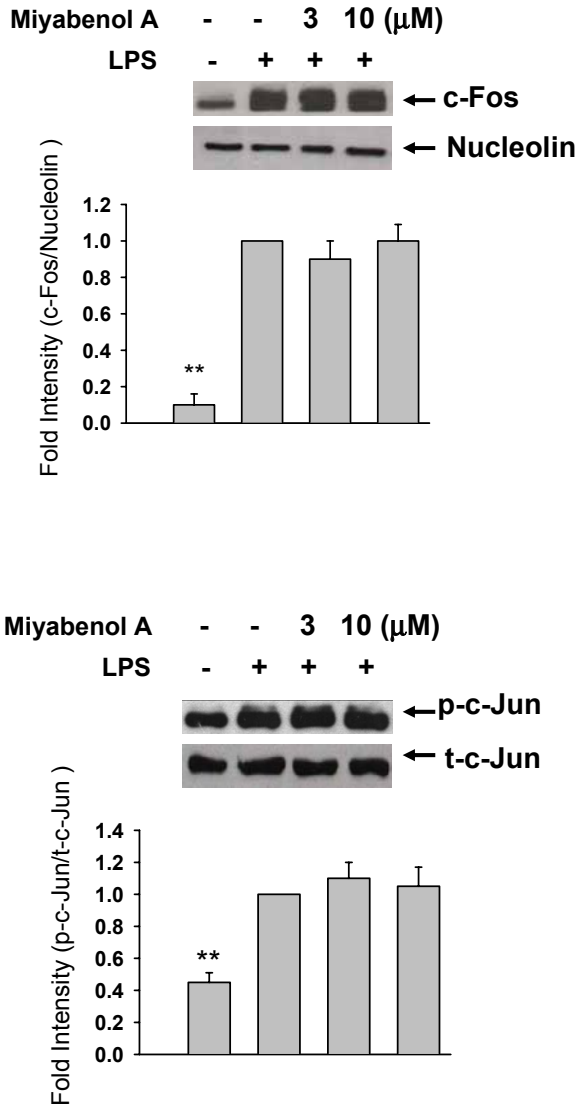

Fig. 2 Effects of miyabenol A on lipopolysaccharide (LPS)-stimulated c-Fos nuclear translocation and c-Jun phosphorylation in RAW264.7 macrophages. Cells were treated with miyabenol A (3 and 10 μM) for 30 min, and then exposed to LPS (1 μg/ml) for 30 min. Nuclear extract and total cell lysate were assessed by Western blotting and protein signals were detected with antibody against total c-Fos and phosphorylated c-Jun, respectively. Data reported are mean ±SE of four independent experiments. ** *P* <0.01, indicate significance of difference as compared with samples receiving LPS alone.

Effect of miyabenol A on LPS-induced JAK/STAT phosphorylation

The phosphor-antibodies were used to determine the activation status of JAK-1/STAT-1. As shown in Figure 3, LPS transcriptionally activated both JAK-1 and $STAT_1$, as revealed by increased phosphorylation level when compared with un-stimulated control. The increased phosphorylations of JAK-1/STAT-1 by LPS were reduced by miyabenol A (3 and 10 μM), but the total forms of JAK-1/STAT-1 remained constant.

Discussion

Miyabenol A is a stilbene isolated from the anti-inflammatory Chinese herb *Vitis thunbergii*. An aberrant expression of iNOS leading to inappropriate NO production has been implicated in human autoimmune and pro-inflammatory diseases. We have reported that miyabenol A displays potent NO inhibitory effect in LPS-stimulated RAW264.7 macrophages [2]. One possible way to inhibit excessive NO production by iNOS is specific enzyme inhibition. However, the use of inhibitors of the iNOS enzyme activity is reported to provoke secondary undesired effects [19, 20]. An alternative approach to block iNOS-dependent NO production is the suppression of iNOS induction [21].

We have provided evidence that suppression of iNOS mRNA and protein expressions might account for the NO inhibitory effect by miyabenol A [2]. In contrast to endothelial NOS and neuronal NOS, which are constitutively expressed, expression of iNOS has to be induced [22]. The transcription factors involved in the regulation of iNOS expression include NF-κB, AP-1, and STAT [22-24].

Current understanding of the use of plant

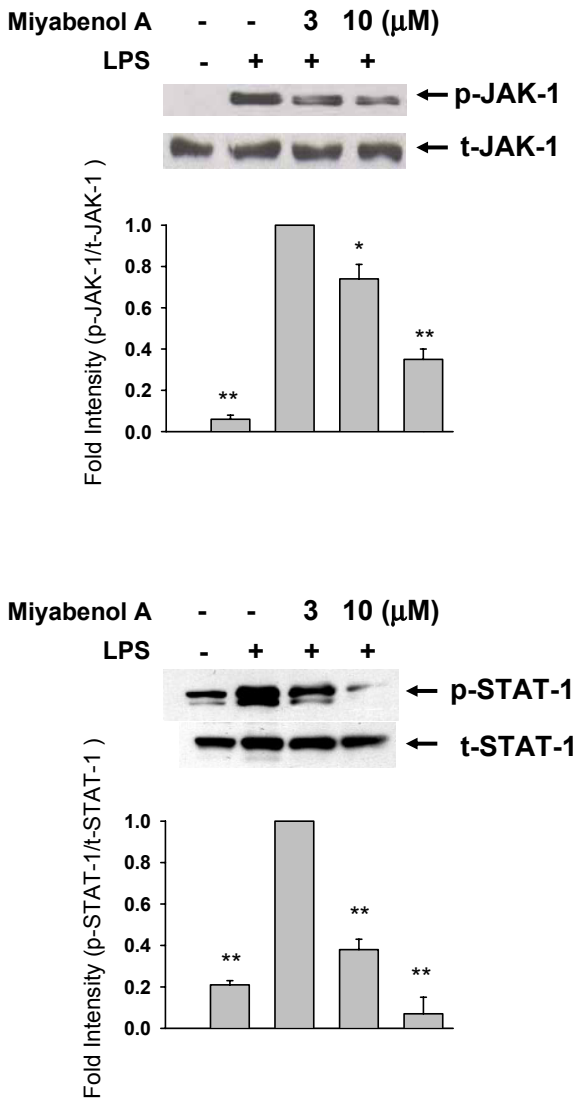

Fig. 3 Effects of miyabenol A on lipopolysaccharide (LPS)-stimulated phosphorylations of JAK-1 and STAT-1 in RAW264.7 macrophages. Cells were treated with miyabenol A (3 and 10 μM) for 30 min, and then exposed to LPS (1 μg/ml) for 30 min. Total cell lysate were assessed by Western blotting and protein signals were detected with antibody against total and phosphorylated JAK-1/STAT-1, respectively. * P<0.05 and ** P <0.01, indicate significance of difference as compared with samples receiving LPS alone.

extracts and/or natural products to alleviate inflammatory diseases concerns their action against the ubiquitous transcription factor, NF-κB. As an activator of many pro-inflammatory cytokines and inflammatory processes, the modulation of the NF-κB transduction pathway is a principal target to alleviate the symptoms of inflammatory diseases. The nuclear translocation and DNA binding of NF-κB is preceded by the degradation of IκB [25, 26]. The present results indeed showed that cell stimulated with LPS evoked a significant IκB-α degradation and the subsequent NF-κB nuclear translocation, and we found that miyabenol A has the ability to inhibit this activation pathway in a concentration-dependent manner.

In addition to NF-kB, AP-1 has been one of the most commonly studied transcription factors that modulate iNOS expression [18]. c-Jun/c-Fos heterodimer is the prominent form of AP-1 [10]. The results that miyabenol A affected neither the c-Jun phosphorylation nor the nuclear level of c-Fos in response to LPS stimulation, suggesting that miyabenol A might inhibit LPS-induced iNOS expression through an AP-1-indpendent signal pathway.

In recent years, several authors described the dependence of human iNOS induction on JAK/STAT pathway [22-24]. To date, four mammalian JAKs and seven mammalian STAT family members have been identified, which may be activated individually or in combination [27, 28]. The divergent and combinational coupling between JAKs and STATs clarify part of the basis for the specificity of signals that are induced by a variety of cytokines. In inducible NO production from macrophage, genetic detection of IFNγ-activated site (GAS), IFN-stimulated responsive element (ISRE), and IFN responsive element (IRE) in iNOS

promoter and functional analysis all indicate STAT-1 as the crucial regulator for iNOS gene transcription [5, 29, 30]. Therefore, the JAK–STAT pathway seems to be a reasonable target for the development of inhibitors of iNOS expression. Measurements of LPS-induced JAK-1/STAT-1 phosphorylation in the present study clearly indicate that miyabenol A may reduce NO production through inhibiting the phosphorylation of JAK-1/STAT-1, consequentially nuclear translocation of STAT-1, and hence transactivation of iNOS gene.

In summary, we have demonstrated that inhibition of LPS-induced NO production by miyabenol A in RAW264.7 cells might be attributed to the down-regulation of NF-κB and JAK-1/STAT-1 pathways probably through the interference with phosphorylation and subsequent nuclear translocation. Further investigation is required to identify the molecular target(s) of miyabenol A in the upstream of NF-κB and JAK/STAT signaling pathways.

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References

1. Chiu NY, Chang KH. The illustrated medicinal plants of Taiwan. Vol. 4. SMC Publisher, Taipei, pp. 147-149, 1995.
2. Ku KT, Huang YL, Huang YJ, Chiou WF. Miyabenol A inhibits LPS-induced NO production via IKK/I κ B inactivation in RAW 264.7 macrophages: possible involvement of the p38 and PI3K pathways. (2008, in press)
3. Petros A, Bennett D,Vallance P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. Lancet 338: 1557-1558, 1991.
4. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6: 3051-3064, 1992.
5. Xie QW, Whisnant R, Nathan C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. J. Exp. Med. 177: 1779-1784, 1993.
6. Giuliani C, Napolitano G, Bucci I, Montani V, Monaco F. NF-κB transcription factor: role in the pathogenesis of inflammatory, autoimmune and neoplastic diseases and therapy implications. Clin. Ther. 152: 249-253, 2001.
7. Stancovski I, Baltimore D. NF-kappaB activation: the I kappaB kinase revealed? Cell 91: 299-302, 1997.
8. Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev. 18: 2195-2224, 2004.
9. Adcock IM. Transcription factors as activators of gene transcription: AP-1 and NF-kappa B. Monaldi Arch. Chest Dis. 52: 178-186, 1997.
10. Hess J, Angel P, Schorpp-Kistner M. AP-1 subunits: quarrel and harmony among siblings. J. Cell Sci. 117: 5965-5973, 2004.
11. Bolli R, Dawn B, Xuan YT. Role of the JAK–STAT pathway in protection against myocardial ischemia/reperfusion injury. Trends Cardiovasc. Med. 13: 72-79, 2003.
12. Schindler C, Darnell Jr JE. Transcriptional response to polypeptide ligands: the JAK-STAT pathway. Annu. Rev. Biochem. 64: 621-651, 1995.
13. Darnell JE, Kerr IM, Stark GR. Jak-STAT

pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415-1421, 1994.

14. Huang YL, Tsai WJ, Shen CC, Chen CC. Resveratrol derivatives from the roots of *Vitis thunbergii*. J. Nat. Prod. 68: 217-220, 2005.
15. Ko HC, Kuo YH Wei BL, Chiou WF. Laxifolone A suppresses LPS/IFN-γ-induced NO synthesis by attenuating NF-κB translocation: role of NF-κB p105 level in RAW264.7 macrophages. Planta Med. 71: 514-519, 2005.
16. Chen CC, Tsai PC, Wei BL, Chiou WF. 8-Prenylkaempferol suppresses inducible nitric oxide synthase expression through interfering with JNK-mediated AP-1 pathway in murine macrophages. Eur. J. Pharmacol. 2008 (in press).
17. Janssen-Heininger YM, Poynter ME, Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. Free Radic. Biol. Med. 28:1317-1327, 2000.
18. Marks-Konczalik J, Chu SC, Moss J. Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor kappa B-binding sites. J. Biol. Chem. 273: 22201-22208, 1998.
19. Husain K. Exercise conditioning attenuates the hypertensive effects of nitric oxide synthase inhibitor in rat. Mol. Cell Biochem. 231:129-137, 2002.
20. Kang DG, Hur TY, Lee GM, Oh H, Kwon TO, Sohn EJ, and Lee HS. Effects of *Cudrania tricuspidata* water extract on blood pressure and renal functions in NO-dependent hypertension. Life Sci. 70: 2599-2609, 2002.
21. Yao Y, Hausding M, Erkel G, Anke T, Forstermann U, and Kleinert H. Sporogen, S14–95 and S-curvularin, three inhibitors of human inducible nitricoxide synthase expression isolated from fungi. Mol. Pharmacol. 63: 383-391, 2003.
22. Kleinert H, Boissel JP, Schwarz PM, and Förstermann U. Regulation of the expression of nitric oxide synthase isoforms. In: Nitric Oxide: Biology and Pathobiology. Ignarro LJ (ed.). Academic Press, New York, pp. 105-128, 2000.
23. Kleinert H, Wallerath T, Fritz G, Ihrig-Biedert I, Rodriguez-Pascual F, Geller DA, and Förstermann U. Cytokine induction of NO Synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-kB-signaling pathways. Br. J. Pharmacol. 125: 193-201, 1998.
24. Dell'Albani P, Santangelo R, Torrisi L, Nicoletti VG, de Vellis J, Giuffrida Stella AM. JAK/STAT signaling pathway mediates cytokine-induced iNOS expression in primary astroglial cell cultures. J. Neurosci. Res. 65:417-424, 2001.
25. Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF kappa B transcription factor. Science 242: 540-546, 1988.
26. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NFkappaB activation. Cell 91: 243-252, 1997.
27. Leonard WJ, O'shea JJ. Jaks and stats: biological Implications. Annu. Rev. Immunol. 16: 293-322, 1998.
28. Seidel HM, LAMB P, Rosen J. Pharmaceutical intervention in the JAK/STT signaling pathway. Oncogene 19: 2645-2656, 2000.

29. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, Murphy WJ. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon g and lipopolysaccharide. Proc. Natl. Acad. Sci. U.S.A. 90: 9730-9734, 1993.

30. Heitmeier MR, Scarim AL, Corbett JA. Prolonged STAT1 activation is associated with interferon-g priming for interleukin-1-induced inducible nitric-oxide synthase expression by islets of Langerhans. J. Biol. Chem. 274: 29266-29273, 1999.

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MIYABENOL A 在RAW 264.7細胞株透過干擾 LPS 誘導之 NF-κB 及 JAK/STAT 途徑而表現出抗發炎活性

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根據我們之前的研究發現分離自小本山葡萄 (Vitis thunbergii) 的 miyabenol A (濃度由 0.1 到 10 μM) 可有效抑制在RAW 264.7細胞株由 LPS 所誘導的一氧化氮 (NO)生成以及誘導型 NO 合成酶 (iNOS)的表現, 本篇實驗進一步在轉錄層面探討 miyabenol A抑制 NO生成的分子作用機轉。以 LPS 刺激細胞可引起 NF-κB轉移入細胞核內同時伴隨細胞質中 IκB的裂解, 這些現象可以被 miyabenol A (3 及 10 μM) 所抑制。 LPS同時也增加 c-Jun 的磷酸化及c-Fos轉移入細胞核內的量 (AP-1的兩個主要組成), 然而 miyabenol A 並不會影響前述活化反應。此外, 由 LPS 所誘導的 JAK-1/STAT-1磷酸化增加也會被 miyabenol A 所抑制。以上結果顯示 miyabenol A 可能透過干擾 LPS 誘導之 NF-κB 及 JAK/STAT 途徑而在 RAW 264.7 細胞株表現出抑制 NO 生成的抗發炎活性。

關鍵詞：小本山葡萄，Miyabenol A，LPS，誘導型 NO合成酶，NF-κB，AP-1，JAK/STAT

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