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Silymarin inhibits macrophage activation

조 선 대 학 교 대 학 원

의 과 학 과

김 은 정

Silymarin inhibits macrophage activation

Silymarin에 의한 대식세포 활성화 억제에 관한 연구

2015 년 2 월 25 일

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Silymarin inhibits macrophage activation

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The present study showed that silymarin, a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum*), inhibited lipopolysaccharide (LPS)-induced morphological changes in the mouse RAW264.7 macrophage cell line. Silymarin inhibited adhesion activity, nitric oxide (NO) production, and inducible nitric oxide synthase gene expression. We also showed that silymarin inhibited the nuclear translocation and transactivation activities of nuclear factor-kappa B (NF-κB), which is important for macrophage activation-associated changes in cell morphology and gene expression of inflammatory cytokines. BAY-11-7085, an NF-κB inhibitor, abrogated LPS-induced morphological changes and NO production, similar to silymarin. Treatment of RAW264.7 cells with silymarin also inhibited LPS-stimulated activation of mitogen-activated protein kinases (MAPKs). Collectively, these experiments demonstrated that silymarin inhibited LPS-induced morphological changes in the RAW264.7 mouse macrophage cell line. Our findings indicated that the most likely mechanism underlying this biological effect involved inhibition of the MAPK pathway and NF-κB activity. Inhibition of these activities by silymarin is a potentially useful strategy for the treatment of inflammation because of the critical

roles played by MAPK and NF-κB in mediating inflammatory responses in macrophages.

국문초록

Silymarin에 의한 대식세포 활성화 억제에 관한 연구

Silymarin은 *Silybum marianum*에서 분리해낸 폴리페놀릭 플라보노이드로 대식세포의 활성화에 대한 영향을 분석하였다. Silymarin은 LPS에 의해 유도된 마우스 대식세포주인 RAW264.7세포의 형태학적 변화, 세포부착활성, NO생성, iNOS유전자 발현을 저해하였다. Silymarin은 또한 염증성 사이토카인 발현에 중요한 NF-κB의 전사촉진과 핵으로의 이동을 억제하였다. NF-κB의 억제제인 BAY-11-7085는 silymarin과 비슷하게 RAW264.7세포의 형태학적 변화와 NO생성을 억제하였다. RAW264.7세포에 silymarin을 처리하면 MAPKs의 활성 또한 저해됨을 확인하였다. 결과적으로 이 연구에서는 대식세포에서 silymarin의 LPS에 의한 형태학적 변화의 억제를 증명했다. MAPK와 NF-κB가 대식세포의 염증반응을 매개하는데 중요한 역할을 하기 때문에 silymarin을 염증 조절에 활용가능 할 것으로 판단된다.

I. Introduction

Silybum marianum, milk thistle, is used in traditional Chinese medicine to soothe the liver and promote bile flow [1]. Silymarin is an extract isolated from the fruit and seeds of *Silybum marianum* [2]. Previous studies showed that silymarin protected against hepatotoxicity caused by a variety of toxins and chemicals including microcystin, ochratoxin, ethanol, phenylhydrazine, and acetaminophen [3-6]. High-performance liquid chromatography (HPLC) and capillary zone electrophoresis analyses have identified the main components of silymarin as silibinin, isosilybin, silydianin, and silychristin [7]. Silibinin, a major polyphenolic flavonoid component of silymarin, has shown a number of additional biological activities such as anti-inflammatory and anti-carcinogenic effects [8-11].

Although the mechanisms underlying the effects of silymarin are largely unknown, possible targets have been suggested. Silibinin and related compounds are direct inhibitors of hepatitis C virus RNA-dependent RNA polymerase, suggesting potential for the treatment of hepatitis C [12]. Isosilybin A was shown to act as a partial agonist of the peroxisome proliferator-activated receptor type gamma, a current therapeutic target in type 2 diabetes and metabolic syndrome [13]. Silymarin has antioxidant activity, scavenging free radicals and modulating antioxidant and

inflammatory enzymes [14, 15]. It has also been reported that mitogen-activated protein kinases (MAPKs) are potential targets of silymarin and silibinin [16, 17]. Silymarin inhibits MAPKs and induces apoptosis in established skin tumors in SENCAR mice [16]. Silibinin prevents the activation of MAPKs and nuclear factor-kappa B (NF-κB) in osteoclast precursors in response to RANKL [18].

Silymarin is a standardized mixture and each commercially available silymarin extract therefore contains fairly similar amounts of compounds [2, 19]. The contents of the silymarin preparations are summarized in Table 1. Silibinin comprises up to 40% of this preparation. The only large difference observed between silymarin products was the greater amount of silydianin in the Indena and Madaus extracts, relative to the Aldrich preparation [2, 19].

A common feature of the major components of silymarin is their flavonolignan skeleton (Fig. 1). Basically, the flavonolignan nucleus consists of the dihydroflavanol, taxifolin, linked to a coniferyl alcohol moiety via an oxeran ring. The oxeran ring is responsible for the biological activities of silymarin [19].

Inflammation is a pivotal process contributing to host defense against pathogens and restoration of a normal structure to damaged tissue [20]. Macrophages are the primary proinflammatory cells and they mediate most

cellular and molecular inflammation processes by producing proinflammatory mediators, including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor-necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) [21]. Macrophages are activated by inflammation-inducing agents, such as growth factors, cytokines, and endotoxins [22, 23]. Once macrophages are activated, they express inducible NO synthase (iNOS) which catalyzes the oxidative deamination of L-arginine and produces NO. Although production of NO is important for host defense against pathogens, continuous generation of NO can cause harmful effects in the pathogenesis of many inflammatory diseases, such as septic shock and rheumatoid arthritis [21]. PGE_2, another important inflammatory mediator, is produced by cyclooxygenases (COXs) [24]. Lipopolysaccharide (LPS), a bacterial endotoxin, induces macrophages to produce inflammatory mediators, such as iNOS, TNF-α, IL-1β, and COX-2 [22, 25].

The gene expression of inflammatory mediators such as iNOS, TNF-α, IL-1β, and COX-2, is regulated by the transcription factor, NF-κB [25, 26]. It has been reported that protein binding to the κB site is necessary to confer LPS inducibility [27]. The promoters of the genes encoding iNOS, TNF-α, IL-1β, and COX-2 contain NF-κB binding sites [28]. NF-κB is present as an inactive form bound to IκB, an inhibitor of NF-κB, in the cytoplasm of unstimulated cells. Stimulation of macrophages induces the

phosphorylation of IκB, resulting in the release of NF-κB. Free NF-κB then translocates to the nucleus, binds to promoter regions, and increases the expression of many genes responsible for the inflammatory process.

LPS stimulation of macrophages induces morphological changes, including an increase in cell size, production of lamellipodia and filopodia, and alterations in cell adhesion, migration, and phagocytosis [29]. Lamellipodia are thin sheets of cell edges containing large amounts of actin filaments and filopodia are actin-containing spikes [30]. Cytoskeletal and adhesion dynamics which mediate migration are regulated by Rho GTPases including RhoA, the Rac subfamily, and Cdc42 [31]. NF-κB also regulates the actin cytoskeleton via integrin-mediated signal transduction, and consequently induces morphological changes in these cells, including lamellipodia formation [32].

Due to the important role of macrophages in inflammation, suppression of their LPS-induced morphological changes and production of inflammatory mediators is an effective way to prevent a variety of inflammatory disorders. A mouse macrophage cell line, RAW 264.7, provides an excellent model to test anti-inflammatory compounds and to investigate the mechanisms related to the inhibition of proinflammatory enzymes and cytokines [33].

Table 1. Composition (% of total material) of commercial milk thistle products

Name	Adrich	Indena	Madaus	Sigma
Silibinin	38.1	34.7	39.8	40.94
Isosilibinin	7	10.5	10.8	3.67
Silychristin	16.5	15.5	11.6	5.31
Silidianin	0.8	9.4	16.7	16.76
Isosilychristin	0.6	1.5	2.2	–
Taxifolin	1.7	2.2	1.6	3.14

*Note that silymarin product composition totals are <100%; each of these products are labeled as 70% to 80% silymarin with the remainder consisting of uncharacterized polyphenols and aliphatic fatty acids such as oleic and palmitic acids.

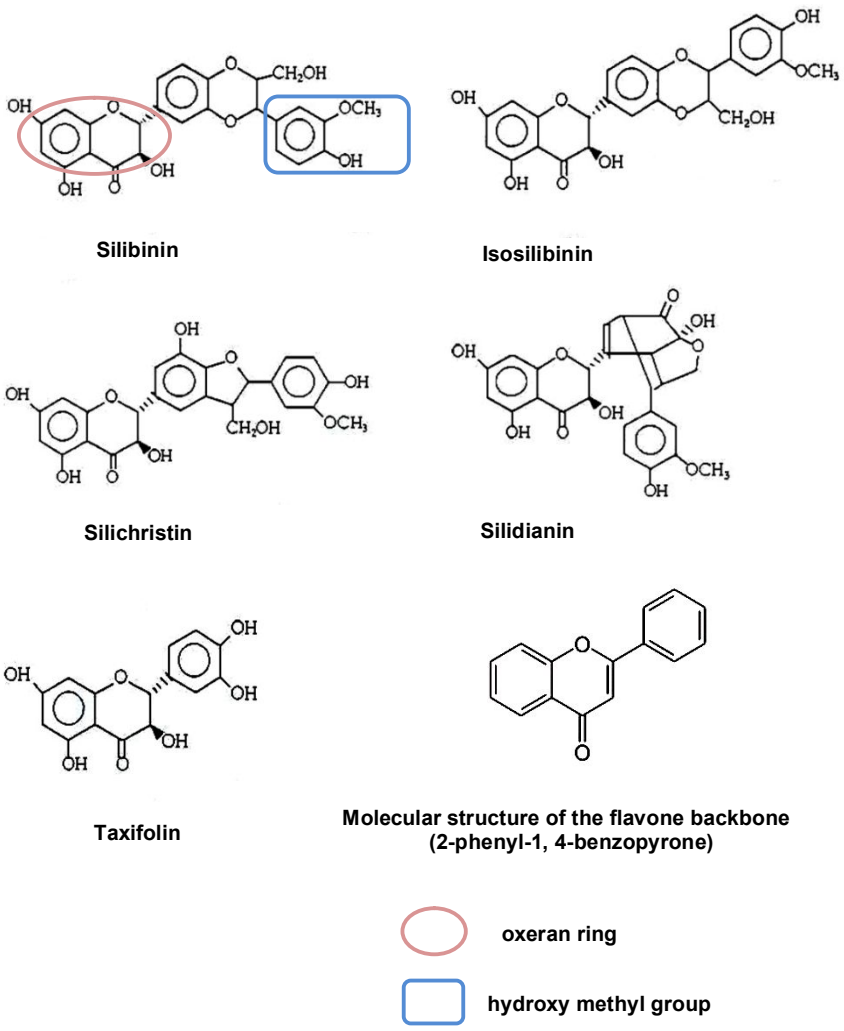


Figure 1. Chemical structures of silymarin components

The chemical structures of the 5 major components of silymarin (silybin, isosilybin, silydianin, silychristin, and taxifolin) are shown.

II. Materials and Methods

II-1. Materials

Silymarin, BAY-11-7085, and LPS from *Salmonella thyposa* were purchased from Sigma (St. Louis, MO). PD98059 (2'-amino-3'-methoxyflavone) was purchased from CalBiochem (San Diego, CA). The anti-iNOS antibody and antibodies against phospho-p44/42 and p44/42 were purchased from Upstate Biotechnology (Lake Placid, NY) and Cell Signaling Technology, Inc. (Beverly, MA), respectively.

II-2. Cell cultures

Raw264.7 cells murine macrophage lines were purchased from American type Culture Collection (Bethesda, MD). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured in the presence of 5% CO_2 at 37℃.

II-3. Morphological analysis

The RAW 264.7 cells (5×10^5 cells/ml) were cultured in the presence of silymarin and LPS for 3, 6, or 12 h on cover slides in 12-well plates.

Morphological changes were analyzed by light microscopy and Giemsa staining. Giemsa's solution is a mixture of methylene blue, eosin, and azure B. It specifically attaches to the phosphate groups of DNA, where there are high amounts of adenine-thymine bonding.

II-4. Cell adhesion assay

The cell adhesion assay was performed as described previously [34], with minor modifications. RAW264.7 cells were treated with silymarin (50 μg/ml) in the presence of LPS (200 ng/ml) for 18 h. Cells were collected and re-plated at a density of 1×10^5 cells/ml. After 30 min, unattached cells were removed by washing with phosphate-buffered saline (PBS) 3 times. Cell adhesion was calculated by counting the attached cells and expressing this number as a percentage of the total cells.

II-5. Nitrite determination

RAW264.7 cells were treated with the indicated concentrations of silymarin in the presence of LPS (200 ng/ml) for 18 h. Culture supernatants were collected, and the accumulation of nitrite in culture supernatants was measured as an indicator of NO production in the medium, as previously described [35, 36].

II-6. Western immunoblot analysis

Whole cell lysates were separated by 10% SDS-PAGE and then electrotransferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were then preincubated for 1 h at room temperature in Tris-buffered saline (TBS), pH 7.6, containing 0.05% Tween-20 and 3% bovine serum albumin, followed by incubation with iNOS, phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), and phospho-ERK1/2 (Thr202/Tyr204)-specific antibodies. Immunoreactive bands were detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagent (Amersham).

II-7. RT-PCR

Total RNA was isolated with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). Forward and reverse primer sequences were as follows: iNOS: 5′-CTG CAG CAC TTG GAT CAG GAA CCT G-3′, 5′-GGG AGT AGC CTG TGT GCA CCT GGA A-3′; and 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 with oligo (dT) 15 primers. PCR was performed with cDNA and each primer.

Samples were heated to 94°C for 5 min and cycled 30 times at 94°C for 1 min, 55°C for 1.5 min, and 94°C for 1 min, after which an additional extension step at 72°C for 5 min was conducted. PCR products were separated by 8% SDS-PAGE, followed by staining with ethidium bromide. The iNOS and β-actin primers produced amplified products of 311 and 349 bps, respectively.

II-8. Luciferase reporter gene assay

RAW264.7 cells were transfected with pNF-κB-Luc plasmid. Cells were incubated for 24 h and treated with silymarin (50 μg/ml) in the presence of LPS for 18 h. Cell extracts were prepared and analyzed luciferase activity for NF-κB transactivation activity.

II-9. Immunoflouoresence staining

RAW264.7 cells were treated with silymarin (50 μg/ml) in the presence of LPS on a cover slide in 12-well plates. Cells were rinsed 3 times with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and rinsed again. Cells were then blocked with 1% bovine serum albumin, followed by the addition of the primary antibody. After extensive washing with Tris-buffered saline, fluorescein isothiocyanate-conjugated IgG was

added. Following incubation, the slides were rinsed, mounted, and viewed at 488 nm on a confocal microscope (FV300, Olympus, Japan).

II-10. Statistical analysis

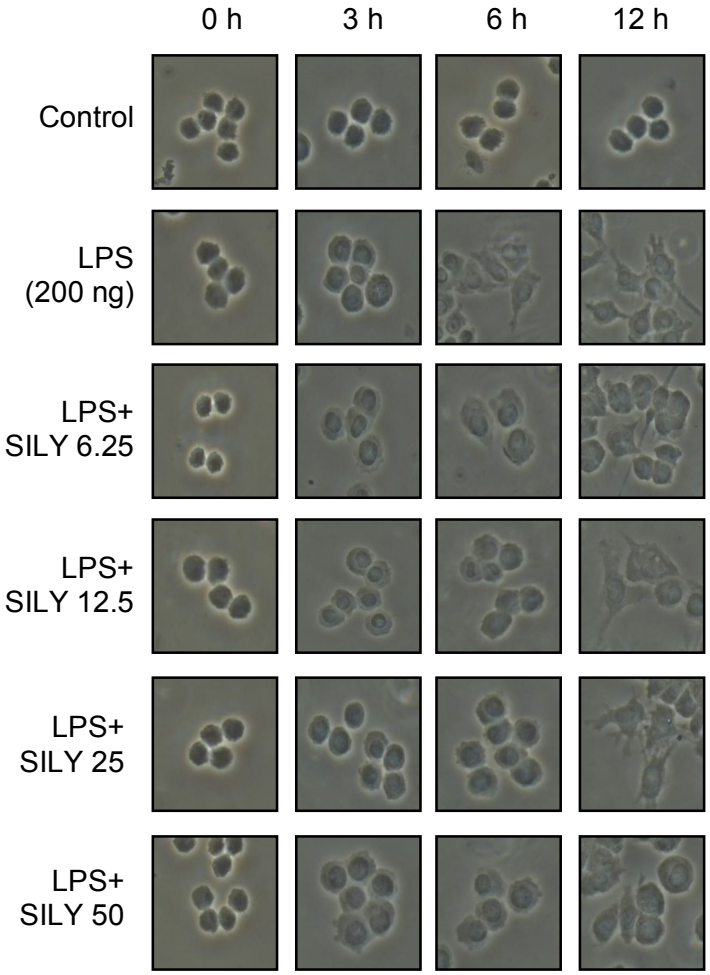
The mean ± SD was determined for each treatment group in a given experiment. When significant differences were present, treatment groups were compared to the respective vehicle controls using a Student's two-tailed *t* test.

III. Results

III-1. Inhibition of macrophage activation by silymarin in LPS-stimulated RAW264.7 cells

To investigate the effects of silymarin on macrophage activation, we used microscopical analysis of LPS-stimulated mouse macrophage cell line RAW 264.7, because LPS-induced morphological change is a hallmark of macrophage activation. The RAW 264.7 cells (5x 10^5 cells/ml) were cultured in the presence of silymarin and LPS for 3, 6, or 12 h on cover slides in 12-well plates. LPS stimulation on macrophages for 6 h and 12 h induced pseudopodia extension and spreading of cells (Fig. 2A). However, unstimulated cells did not show morphological changes at any time point. Silymarin treatment of macrophages prevented LPS-induced morphological changes at 6 h and 12 h in a dose-dependent manner (Fig. 2A). Fully spread cell counts further showed silymarin inhibited LPS-induced morphological changes of macrophages (Fig. 2B). These data suggest that silymarin inhibits macrophage activation in LPS-stimulated RAW 264.7 cells.

A



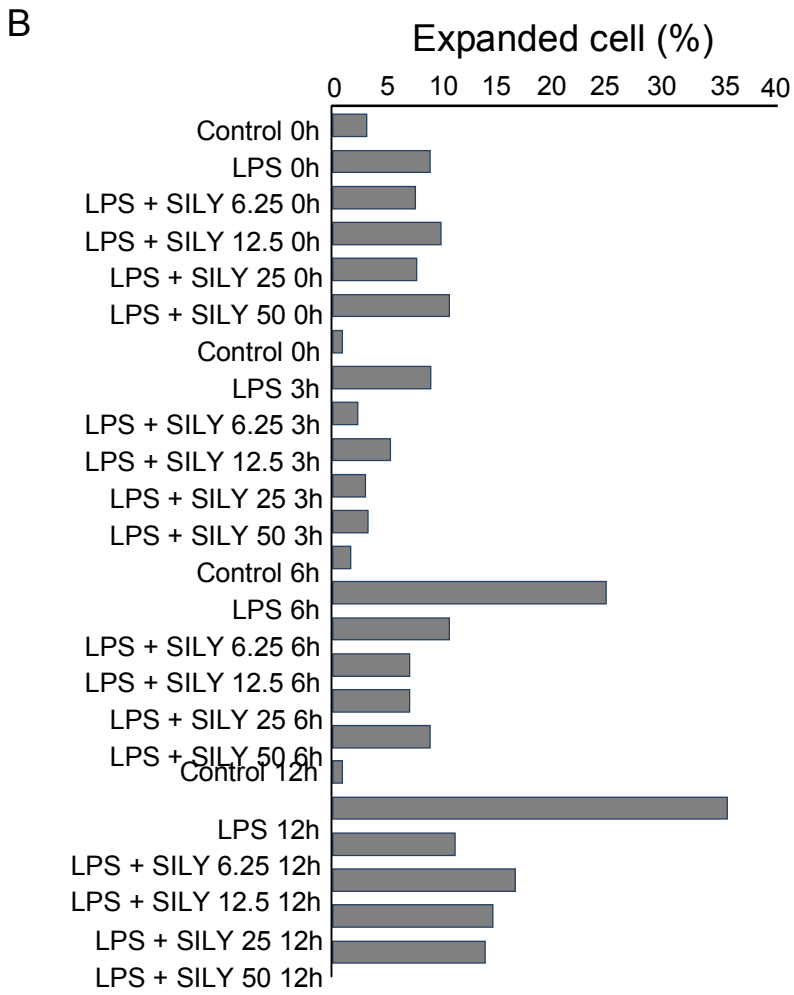


Figure 2. Inhibition of macrophage activation by silymarin in lipopolysaccharide (LPS)-stimulated macrophages

RAW264.7 cells were treated with the indicated concentrations of silymarin in the presence of LPS (200 ng/ml) for 3 h, 6 h, or 12 h on cover slides in 12-well plates. Cells were then subjected to microscopic analysis. (A) Representative photographs. (B) Fully expanded cells, expressed as a percentage of the total number of cells.

III-2. Inhibition of morphological changes and adhesion activity by silymarin in LPS-stimulated macrophages

Giemsa staining of RAW264.7 cells treated with LPS for 6 h showed that lamellipodia were extended from 3-5 sides of the cells, and that silymarin inhibited pseudopodia formation (Fig. 3). To further analyze whether the inhibition of lamellipodia formation by silymarin was related to cell adhesion activity, we investigated the effects of silymarin on cell attachment to culture plates. LPS stimulation significantly increased RAW264.7 adhesion, whereas silymarin inhibited this activity (Fig. 4A). Microscopic analysis showed that LPS-stimulated macrophages were attached after washing, while control and silymarin treated macrophages were washed off the plate (Fig. 4B). These results demonstrated that silymarin inhibited both the morphological changes and functional activation of mouse RAW264.7 macrophages.

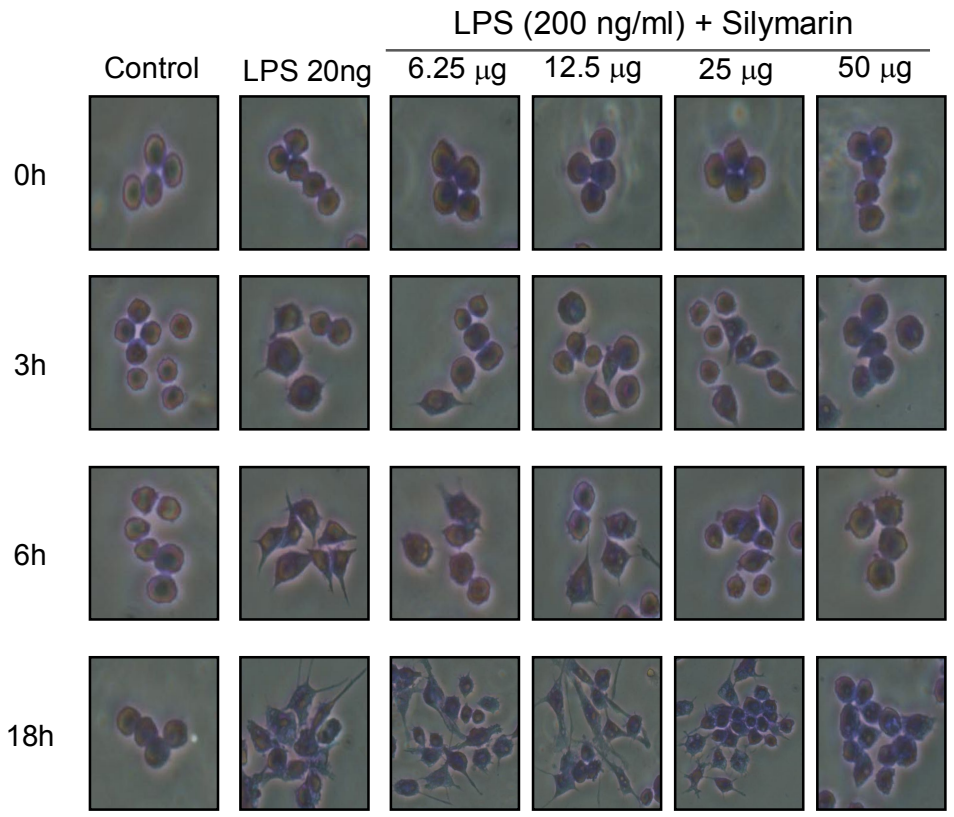
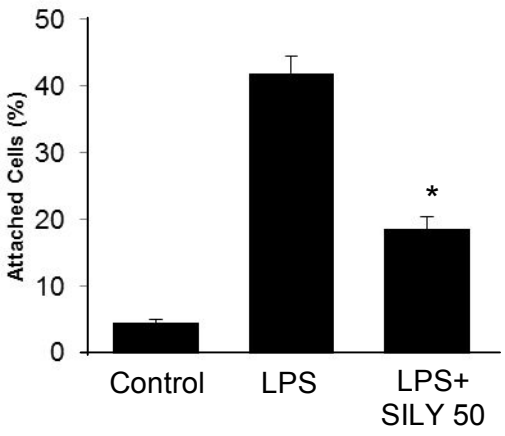


Figure 3. Inhibition of morphological changes by silymarin in lipopolysaccharide (LPS)-stimulated macrophages

RAW264.7 cells were treated with the indicated concentrations of silymarin in the presence of LPS (200 ng/ml) for 3 h, 6 h, or 18 h on cover slides in 12-well plates. Cells were then Giemsa-stained and analyzed for morphological change.

A



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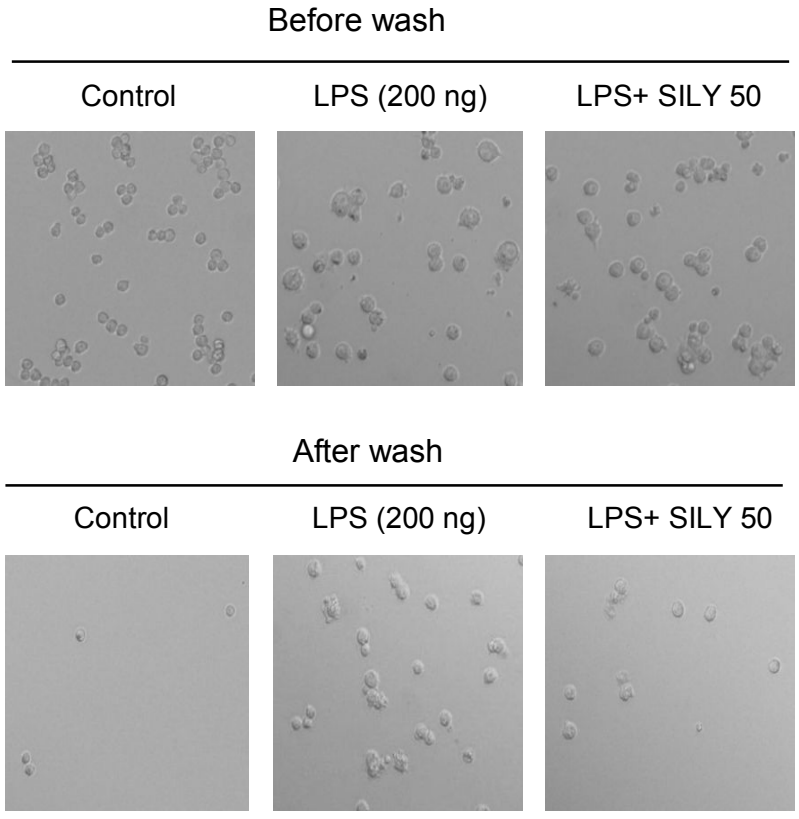

Figure 4. Inhibition of lipopolysaccharide (LPS)-stimulated macrophage adhesion by silymarin

(A) RAW264.7 cells were treated with silymarin (50 μg/ml) in the presence of LPS for 24 h. Cells were harvested, washed, plated in 6-well plates (5×10^5 cells/ml) for 30 min, washed, and analyzed for adhesion using microscopy. Attached cells were counted before and after washing. (B) Representative photographs of attached cells. Each column shows the mean ± SD of triplicate determinations. $*P < 0.05$ compared to the control group, as determined by Student's two-tailed *t* test.

III-3. Inhibition of nitrite production and iNOS expression by silymarin in LPS-stimulated macrophages

We investigated the effects of silymarin on macrophage activation in LPS-stimulated RAW264.7 mouse macrophages using an NO production assay. Macrophages play an essential role in host defense against microbial infection through the generation of variety of chemicals, including NO [37, 38]. LPS-induced generation of nitrite was inhibited by silymarin in a dose-dependent manner (Fig. 5A). MTT assay showed that the viability of all treated cells exceeded 80%, although 25 and 50 μg/ml silymarin treatment slightly inhibited cellular tetrazolium reducing activity (Fig. 5B). Western immunoblot and RT-PCR analyses showed that silymarin inhibited iNOS gene expression, as shown in Fig. 5C and 5D. These results indicated that silymarin decreased expression of iNOS, which is involved in inflammation [37].

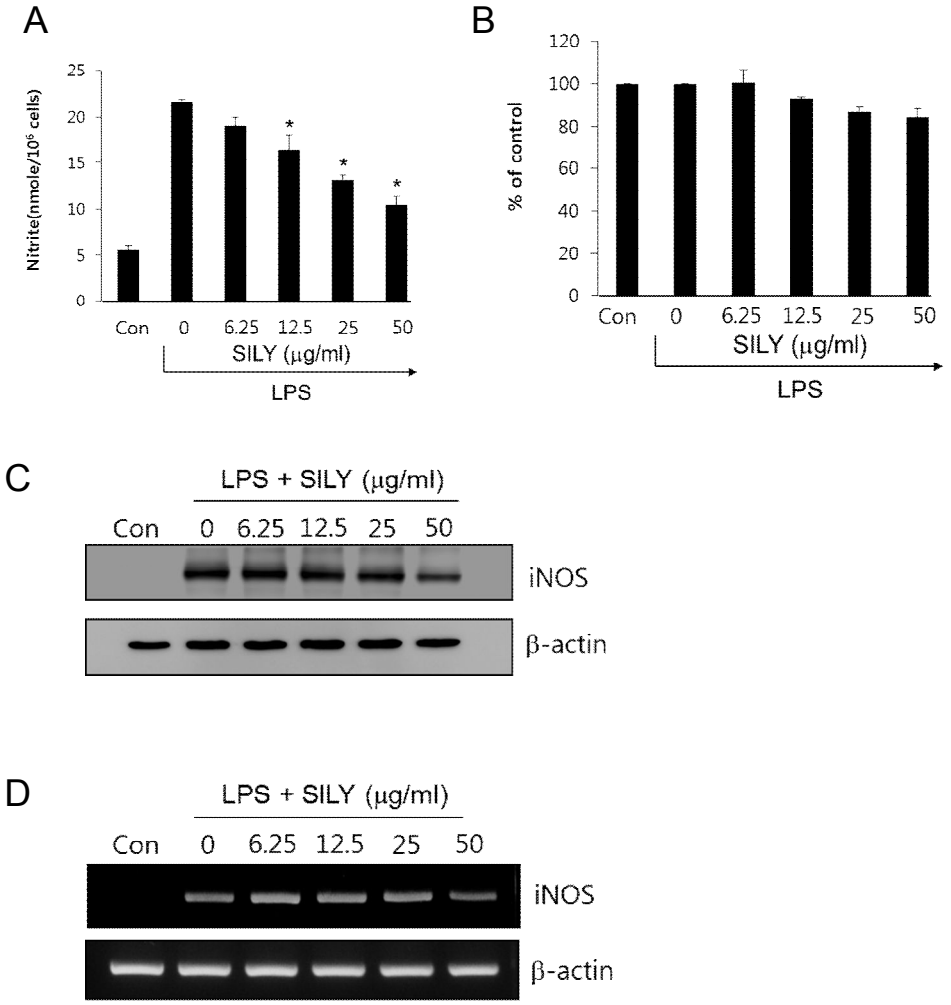

Figure 5. Inhibition of the production of nitrite and iNOS by silymarin in lipopolysaccharide (LPS)-stimulated macrophages

RAW264.7 cells were treated with the indicated concentrations of silymarin in the presence of LPS for 24 h. (A) Supernatants were subsequently isolated and analyzed for nitrite. (B) Cells were analyzed for viability by MTT assay. (C, D) Cells were treated with the indicated concentrations of silymarin in the presence of LPS for 24 h (C) or 8 h (D). (C) Expression of inducible nitric oxide synthase (iNOS) was analyzed by Western blot using an antibody specific for murine iNOS. (D) Total RNA was isolated and analyzed for mRNA expression levels of iNOS and β-actin. Each column shows the mean ± SD of triplicate determinations. $*P < 0.05$ compared to the control group, as determined by Student's two-tailed *t* test.

Ⅲ-4. Inhibition of the NF-κB activation and p65 nuclear translocation by silymarin in LPS-stimulated macrophages

The effect of silymarin on NF-κB, which binds the iNOS gene promoter [37], was evaluated using a luciferase reporter assay. Treatment of RAW264.7 cells with LPS induced a marked increase in NF-κB-induced luciferase activity, whereas the induction of NF-κB activity was inhibited by silymarin (Fig. 6A). Macrophage stimulation induces nuclear translocation of p65, one of the major components of NF-κB, which binds to the promoter and induces transcription. We therefore analyzed the effect of silymarin on the nuclear translocation of p65 using immunofluorescence staining. When RAW264.7 cells were stimulated by LPS for 2 h, p65 levels increased in the nucleus, whereas p65 was mainly localized in the cytosol of unstimulated control cells (Fig. 6B). This LPS-induced nuclear translocation of p65 was inhibited by silymarin. These results indicated that silymarin reduced the nuclear translocation and transactivation activity of NF-κB, which is important in LPS-mediated regulation of inflammatory gene expression in macrophages.

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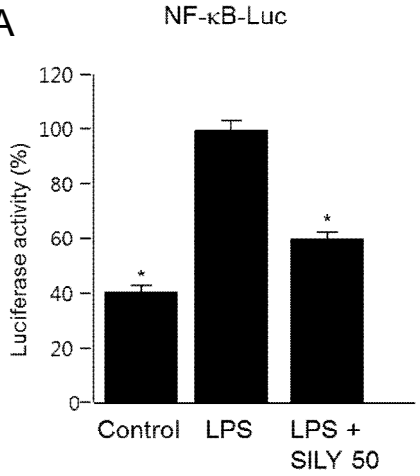

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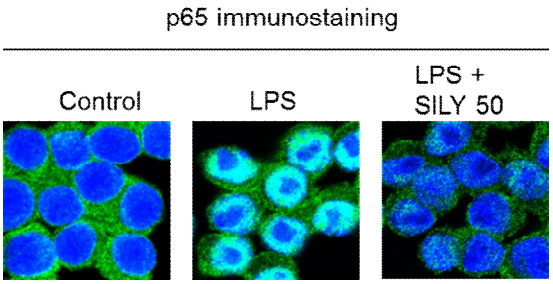

Figure 6. Inhibition of nuclear factor-kappa B (NF-κB) activation and p65 nuclear translocation by silymarin in lipopolysaccharide(LPS)-stimulated macrophages

(A) RAW264.7 cells were transfected with pNF-κB-Luc plasmid. Cells were incubated for 24 h and then treated with silymarin (50 μg/ml) in the presence of LPS for 18 h. Cell extracts were prepared and luciferase activity was analyzed to indicate NF-κB transactivation activity. (B) RAW264.7 cells were treated with silymarin (50 μg/ml) in the presence of LPS for 2 h on cover slides in 12-well plates. Cells were then subjected to immunofluorescence staining of p65 and DAPI staining.

III-5. Inhibition of the NF-κB morphological changes in LPS-stimulated macrophages

To further confirm the involvement of NF-κB activation in the morphological changes, we analyzed the effects of a pharmacological inhibitor of NF-κB on macrophage activation using microscopy and a nitrite generation assay. BAY-11-7085, an inhibitor of IκB phosphorylation (Fig. 7A) inhibited LPS-induced nuclear translocation of p65 (Fig. 7B). Treatment of RAW264.7 cells with BAY-11-7085 significantly inhibited LPS-induced nitrite production and morphological changes in a dose dependent manner (Fig. 7C, 7D). These results demonstrated that NF-κB was important for both the morphological and functional activation of macrophages.

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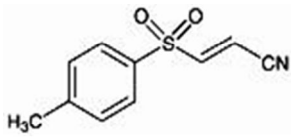

BAY 11-7085

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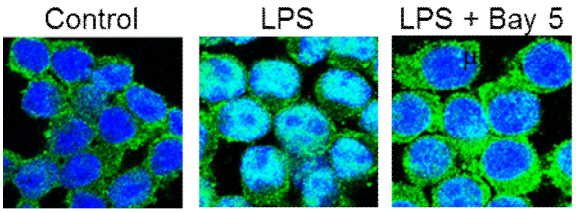

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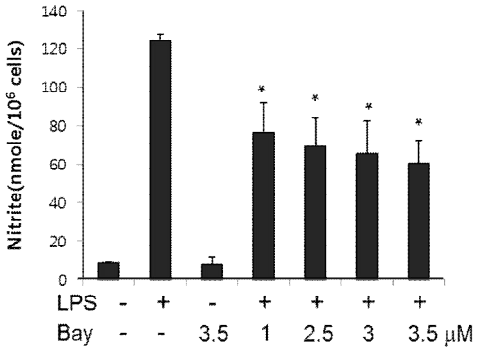

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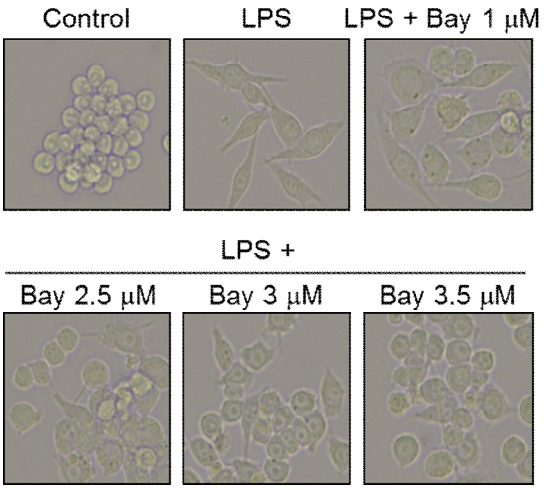


Figure 7. Effects of nuclear factor-kappaB (NF-κB) inhibitor on macrophage activation induced by lipopolysaccharide (LPS)

(A) Structure of BAY-11-7085. (B) RAW264.7 cells were treated with BAY-11-7085 (5 μM) in the presence of LPS for 2 h on cover slides in 12-well plates. Cells were then subjected to immunofluorescence staining of p65 and DAPI staining. (C) RAW264.7 cells were treated with the indicated concentrations of BAY-11-7085 in the presence of LPS for 24 h. Supernatants were subsequently isolated and analyzed for nitrite. Each column shows the mean ± SD of triplicate determinations. $*P < 0.05$ compared to the control group, as determined by Student's two-tailed *t* test. (D) RAW264.7 cells were treated with the indicated concentrations of BAY-11-7085 in the presence of LPS (20 ng/ml) for 6 h on cover slides in 12-well plates. Cells were then subjected to microscopic analysis.

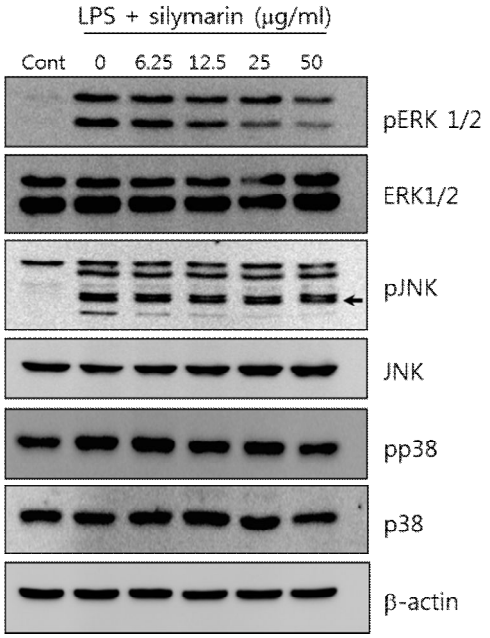
III-6. Inhibition of MAPKs phosphorylation by silymarin in LPS-stimulated RAW264.7 cells

Since MAPKs are important for NO generation in LPS-stimulated RAW264.7 and are potential targets of silymarin, we further determined the role of MAPKs in the inhibition of NO by silymarin. RAW264.7 cells were pretreated with silymarin for 1 h and then incubated for 30 min in the presence of LPS. MAPK phosphorylation was analyzed by Western blot. The phosphorylation of ERK1/2 was strongly increased by LPS treatment, whereas basal ERK1/2 phosphorylation was undetectable (Fig. 8A). When cells were treated with silymarin in the presence of LPS, ERK1/2 phosphorylation was decreased in a dose-dependent manner (Fig. 8B). The phosphorylation of c-Jun N-terminal kinase (JNK) was also increased by LPS and decreased by silymarin (Fig. 8C). Western blot of p38 showed modest basal phosphorylation, slight induction by LPS, and inhibition by silymarin (Fig. 8C).

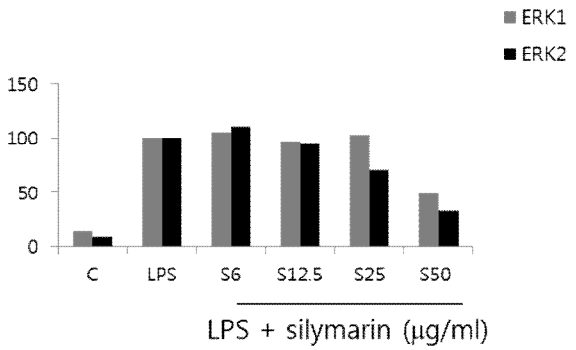
The ERK1/2 kinase pathways were specifically blocked when RAW264.7 cells were treated with LPS. PD98059 is a specific inhibitor of MAPK/extracellular signal-regulated kinase 1 (MEK-1), which is responsible for ERK1/2 activation. PD98059 inhibited LPS-induced production of nitrite, whereas SB203580, a bicyclic inhibitor of p38, had no inhibitory effect (Fig. 9). These results suggested that the ERK1/2 kinase

pathway played an important role in the regulation of NO generation in LPS-stimulated RAW264.7 cells, and that this was inhibited by silymarin.

A



B



C

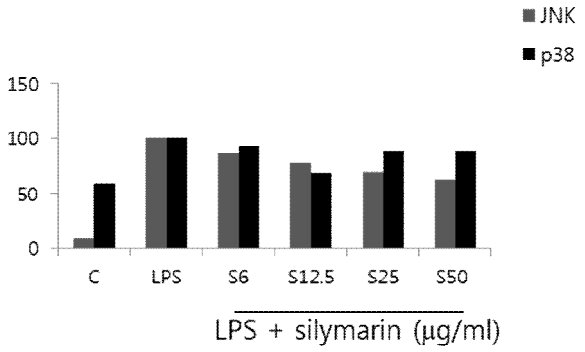


Figure 8. Inhibition of mitogen-activated protein kinase (MAPK) phosphorylation by silymarin in lipopolysaccharide (LPS)-stimulated RAW264.7 cells

RAW264.7 cells were pretreated with silymarin for 1 h and incubated for 30 min in the presence of LPS. The phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2, A), p38 (B), and phospho-c-Jun N-terminal kinase (pJNK, C) was analyzed by Western blot. The relative band densities were analyzed using the Image J program. When cells were treated with silymarin for 20 min in the presence of LPS, the phosphorylation of ERK1/2 was decreased in a dose-related manner.

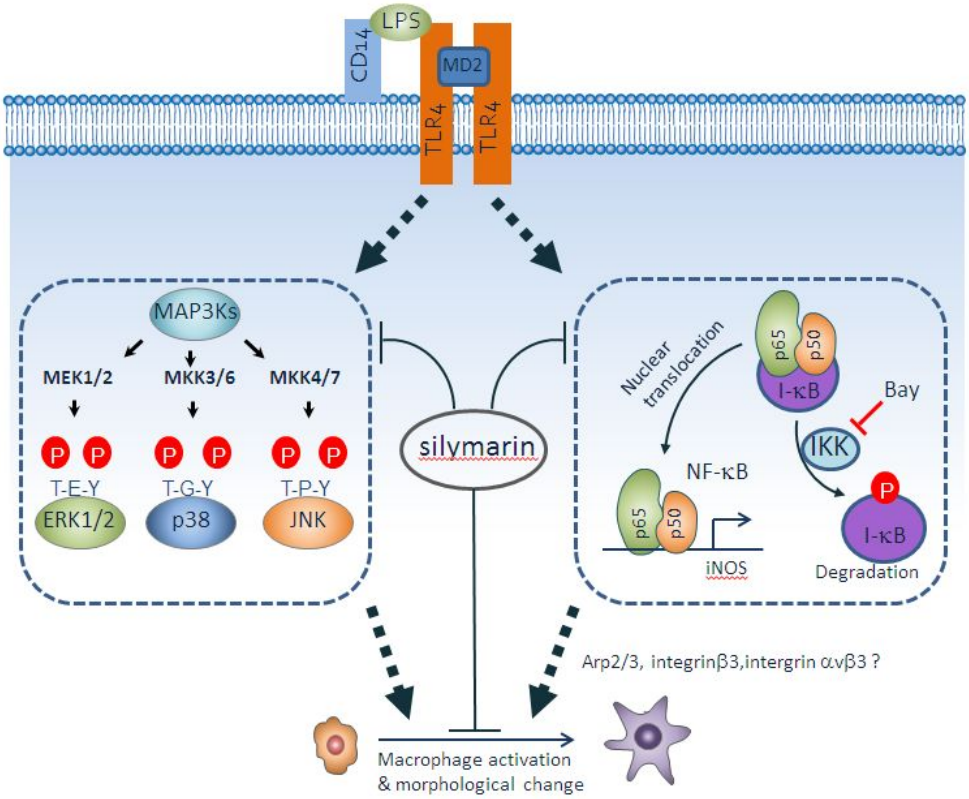


Figure 9. Cartoon showing the signal transduction and possible targets of silymarin

Lipopolysaccharide (LPS) binding to CD14 and TLR4 induces signal transduction pathways, including mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB). The possible targets of silymarin are shown.

IV. Discussion

In the present study, we showed silymarin, a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum*) is a potent anti-inflammatory agent. Silymarin inhibits morphological changes such as lamellipodia and filopodia in LPS-stimulated RAW264.7 cells. Lamellipodia and filopodia are thin-sheets of cell edges containing large amounts of actin filaments and actin-containing spikes, respectively [30]. These morphological changes are the hall marks of inflammatory response of macrophages, because lamellipodia and filopodia formation is important for attachment to extracellular matrix, which is required for cell adhesion and migration to the inflammatory sites. The anti-inflammatory effect of silymarin was further supported by inhibition of NO production, an important mediator of inflammatory responses including phagocytosis [9]. The inhibition of NO production is related to the suppression of iNOS gene expression. Due to the important role of macrophages in inflammation and pathogenesis of autoimmune diseases, then inhibition of morphological and functional changes of macrophages by silymarin represents its application to inflammatory agent. For example, researchers including us demonstrated that silymarin has a protective effect on pancreatic beta cell destruction induced by proinflammatory cytokines [33, 39]. Proinflammatory cytokines

are known to induce the expression of iNOS mRNA and production of NO, resulting in cell death of beta cells [40, 41] An *in vivo* study using a rat model further showed that silymarin increased insulin gene expression and beta cell proliferation [42].

Since lamellipodia and filopodia formation is also important in cancer cell migration and metastasis [43], silymarin may be useful as a chemopreventive agent. Anti-tumor effects of silymarin have been reported. [44-47]. Silymarin and its components, isosilibin and silibinin inhibited advance human prostate cancer growth in athymic nude mice [44]. Silymarin inhibits cervical cancer cell through an increase of phosphatase and tensin homolog (PTEN) [45]. Silymarin also inhibited the phosphorylation of Akt. Silymarin inhibits azoxymethane-induced colon carcinogenesis in male F344 rats [46]. Taken together, these results show that silymarin has the potential to be developed as a promising agent for the treatment of several cancers including prostate, cervical, colon cancer in the future.

One of the most important findings in the present study is the involvement of NF-κB in the morphological changes induced by LPS in macrophages. We showed that silymarin reduces nuclear translocation of p65, a component of NF-κB. In unstimulated cells, heterodimer of p65 and p50, another component of NF-κB, is bound to IκB, an inhibitor protein of

NF-κB. NF-κB released by LPS-induced phosphorylation of IκB, translocate into nucleus. Because p65 has transactivation activity, the nuclear translocation of p65 into the nucleus is a key process of activation of NF-κB. Further silymarin inhibited the NF-κB-dependent luciferase reporter gene activities. The relationship between NF-κB and morphological changes were suggested by our inhibitor study. BAY 11-7085, an inhibitor of IκB phosphorylation, inhibited the nuclear translocation of p65 and morphological changes in LPS-stimulated macrophages. The role of NF-κB in morphological changes is further supported by previous reports [32]. NF-κB regulates the actin cytoskeleton and induces the morphological changes including lamellipodia formation [32]. NF-κB also plays an important role in gene expression of inflammatory mediators including iNOS, COX-2, and cytokines [25, 26]. Our data also showed that silymarin and NF-κB inhibitor prevent NO production. The possible targets of NF-κB in mediating morphological changes are cell adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin [48-50]. Silymarin was found to inhibit monocyte adhesion to endothelial cells and gene expression of cell adhesion molecules on endothelial cells when monocytes were activated [51].

To investigate the mechanism by which silymarin inhibits macrophage

activation, we analyzed the effects of silymarin on the mitogen-activated protein kinases (MAPK) pathways. Macrophage stimulation by external stimuli including LPS induces the phosphorylation of MAPK family, which includes extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) [52]. ERK1/2, p38 MAPK, and JNK are serine threonine kinases that are located in the cytoplasm until activated by dual phosphorylation of their Thr and Tyr residues at Thr-Glu-Tyr, Thr-Gly-Tyr, or Thr-Pro-Tyr, respectively [53]. MAPK pathways are summarized in Fig. 10. We showed that silymarin inhibits ERK1/2, JNK and p38 phosphorylation. ERK2 phosphorylation was most strongly inhibited by silymarin (Fig. 8). It was also showed that MAPKs are potential targets of silymarin and silibinin [16, 18]. Silymarin inhibits growth and causes regression of skin tumors in SENCAR mice through the inhibition of MAPKs [16]. Silibinin prevents the activation of ERK, p38, JNK, and NF-κB in osteoclast precursor cells in response to RANKL [18]. Silibinin inhibits TPA-induced MMP-9 expression through the Raf/MEK/ERK pathway in thyroid and breast cancer cells [54, 55]. Silibinin also prevented TNF-α-induced MMP-9 expression in gastric cancer cells through inhibition of the MAPK pathway [17]. ERK activity is required for iNOS gene expression in insulin-producing INS-1E cells and RAW264.7 cells [56, 57]. Because many researchers including us showed MAPKs

phosphorylation is inhibited by silymarin, the direct targets of silymarin need to be discovered. The upstream kinases including MEK1/2, MKK3/6, and MKK4/7 could be candidates for the targets of silymarin and should be tested first, although we could not exclude the possibilities that MAP3Ks are silymarin's target enzyme.

In the present study, we demonstrated that silymarin inhibits MAPK pathways and NF-κB in LPS stimulated macrophages (summarized in Fig. 10). Although MAPKs and NF-κB plays in pivotal roles in macrophages activation including lamellipodia and filopodia formation, the exact process between these pathways and morphological changes are largely unknown. However, some proteins that control lamellipodia and filopodia formation are known. The complex of actin-related proteins 2/3(Arp2/3) is a major initiator of new actin filaments in cells and plays an essential role in the assembly of actin networks in lamellipodia. Filopodia are controlled by proteins such as fascin, diaphanous, and Mena/ Vasodilator-stimulated phosphoprotein (VASP). Fascin is an actin bundling protein that brings together filaments in lamellipodial networks to encourage filopodial growth [58]. Its actin bundling activity is regulated by phosphorylation and by the small GTPases Rac and Cdc42 [30, 59, 60]. Murine diaphanous is involved in different activities of cytoskeleton including interaction with microtubules [61]. Mena/VASP proteins have the ability to promote the elongation of

long parallel bundles of actin in filopodia [62]. All of these proteins have been implicated in lamellipodial and filopodial actin dynamics, so it seems that silymarin controls these proteins directly or indirectly through the regulation of MAPKs and NF-κB pathways.

Collectively, silymarin is a potent control agent in pathophysiological processes during inflammatory response through the inhibition of MAPKs and NF-κB pathways. Due to the critical roles of the pathways in mediating adhesion and migration of cancer cells, silymarin represents possible chemotherapeutic agents.

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VI. 감사의 글