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Silibinin Inhibits LPS-Induced Macrophage Activation by Blocking p38 MAPK in RAW 264.7 Cells

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Abstract

We demonstrate herein that silibinin, a polyphenolic flavonoid compound isolated from milk thistle (*Silybum marianum*), inhibits LPS-induced activation of macrophages and production of nitric oxide (NO) in RAW 264.7 cells. Western blot analysis showed silibinin inhibits iNOS gene expression. RT-PCR showed that silibinin inhibits iNOS, TNF-α, and IL1β. We also showed that silibinin strongly inhibits p38 MAPK phosphorylation, whereas the ERK1/2 and JNK pathways are not inhibited. The p38 MAPK inhibitor abrogated the LPS-induced nitrite production, whereas the MEK-1 inhibitor did not affect the nitrite production. A molecular modeling study proposed a binding pose for silibinin targeting the ATP binding site of p38 MAPK (1OUK). Collectively, this series of experiments indicates that silibinin inhibits macrophage activation by blocking p38 MAPK signaling.

Key Words: Silibinin, Macrophages, p38 MAPK, Nitric oxide

INTRODUCTION

Silibinin is the major active constituent of silymarin, a standardized extract isolated from the fruit and seeds of the milk thistle, *Silybum marianum* (Pliskova *et al.*, 2005). It is well known that silymarin and silibinin protect against hepatotoxicity caused by a variety of agents including ethanol, phenylhydrazine, acetaminophen, microcystin, and ochratoxin (Mereish *et al.*, 1991; Valenzuela and Garrido, 1994; Al-Anati *et al.*, 2009). Various studies also indicate that silibinin exhibits anticarcinogenic effects (Hogan *et al.*, 2007; Mokhtari *et al.*, 2008). Moreover, silibinin possesses a number of additional biological effects such as anti-inflammatory effects (Kang *et al.*, 2002; Cristofalo *et al.*, 2013). Although the mechanism or mechanisms of action are largely unknown, silymarin and silibinin have been shown to have direct antioxidant activity mediated through the scavenging of free radicals, and modulations of antioxidant and inflammatory enzymes (Letteron *et al.*, 1990; Zhao *et al.*, 1999).

Macrophage stimulation by external stimuli including lipopolysaccharides, a cell wall component of gram-negative bacteria, results in the phosphorylation of mitogen-activated protein kinases (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) (Su and Karin, 1996). 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-Gly-Tyr, Thr-Glu-Tyr, or Thr-Pro-Tyr, respectively (Raingeaud *et al.*, 1995). MAPK activation then stimulates transcription factors including NF-κB and AP-1 (Weinstein *et al.*, 1992; Whitmarsh and Davis, 1996). In particular, the p38 MAPK is an important mediator of stress-induced gene expression and plays a key role in LPS-induced signal transduction pathways leading to cytokine synthesis (Raingeaud *et al.*, 1995; Lee and Young, 1996). The p38 MAPK activation was reported to be involved in iNOS expression in tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1)-stimulated mouse astrocytes, as well as in LPS-stimulated mouse macrophages (Da Silva *et al.*, 1997; Chen and Wang, 1999). It has been shown that MAPKs are potential targets of silymarin and silibinin (Singh *et al.*, 2002; Kim *et al.*, 2009). Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice by modulation of MAPKs and induction of apoptosis (Singh *et al.*, 2002). Silibinin blocks the activation of NF-κB, JNK, p38 MAPK, and ERK in osteoclast precursors in

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response to RANKL (Kim *et al.*, 2009).

In the study presented herein, we investigated the effect of silibinin on macrophage activation and production of NO, an important indicator of inflammation. To further investigate the mechanism by which silibinin inhibits activation of macrophage, we examined the possibility that silibinin targets p38 MAPK for its anti-inflammatory effects. The present study demonstrates that silibinin inhibits macrophage activation through the inhibition of p38 MAPK pathways.

MATERIALS AND METHODS

Materials

Silibinin and LPS from *Salmonella thyposa* was purchased from Sigma (St. Louis, MO, USA). Reagents used for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). PD98059 (2'-amino-3'-methoxyflavone) and SB203580 (4-(4-fluorophe-nyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole) were purchased from Calbiochem (San Diego, CA, USA). Anti-iNOS was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies (anti-phospho-ERK1/2 antibody, anti-phospho p38 MAPK antibody, and anti-JNK antibody) were purchased from New England Biolabs Inc. (Beverly, MA, USA).

Cell culture

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

Nitrite determination

RAW 264.7 cells were treated with the indicated concentrations of silibinin in the presence of LPS (200 ng/ml) for 18 h. Culture supernatants were collected, and the accumulation of NO_2^- in culture supernatants was measured as an indicator of NO production in the medium as previously described (Green *et al.*, 1982; Huong *et al.*, 2012).

Western immunoblot analysis

Whole cell lysates were separated by 10% SDS-PAGE, then electro-transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were 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. The nitrocellulose membranes were then incubated with primary antibodies. Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham).

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'; 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', 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 and visualized by staining with ethidium bromide.

Molecular modeling

The p38 MAPKα (PDB ID: 1OUK) was chosen for docking studies. Its X-ray diffraction structure had a resolution of 2.5 a° (Fitzgerald *et al.*, 2003) and pyridinylimidazole inhibitor was bound to the ATP-binding site of the p38 MAPK. p38 MAPK was prepared for docking using the Protein Preparation Wizard in the Schrödinger Suite 2010 by a standard procedure. Silibinin was prepared using MacroModel of Schrödinger and minimized, and the lowest energy conformations for docking were determined by using default parameters. The protein-ligand docking analysis was conducted using the Induced Fit docking program of Schrödinger, which can provide the ligand binding flexibility with the binding pocket residues. Images were generated using the UCSF Chimera program (Pettersen *et al.*, 2004).

Statistical analysis

The mean ± SD was determined for each treatment group in a given experiment. When significant differences occurred, treatment groups were compared to the vehicle control using a Dunnett's two-tailed *t* test (Dunnett, 1955).

RESULTS

Effect of silibinin on morphological change of macrophage

To investigate the effects of silibinin on macrophage activation, we analyzed the morphological changes in the mouse macrophage cell line, RAW 264.7 induced by LPS. The RAW 264.7 cells (5×10^5 cells/ml) were incubated with silibinin in the

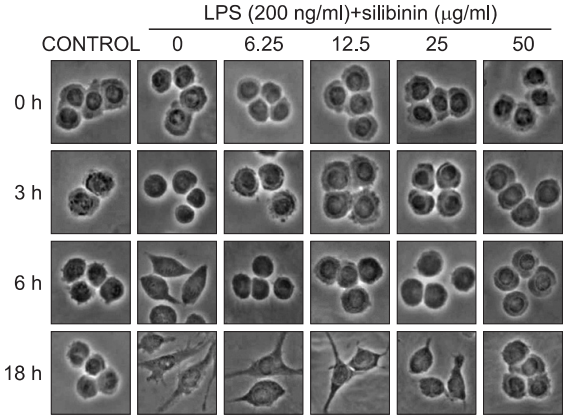

Fig. 1. Inhibition of macrophage activation by silibinin. RAW 264.7 cells (5×10^5 cells/ml) were incubated with silibinin in the presence of LPS (200 ng/ml) for the indicated time on cover slides in 12 well plates. Cells were then subjected to microscopic analysis.

presence of LPS (200 ng/ml) for 3, 6, or 18 hours on cover slides in 12 well plates. When RAW 264.7 cells were exposed to LPS for 6 hours, pseudopodia extended from one or two sides of the cells (Fig. 1). More pseudopodia were extended at 18 hours of LPS-stimulated macrophages. Whereas untreated cells did not show these morphological changes at any time point. Treatment with silibinin inhibited LPS-induced morphological changes of macrophage at 18 h in a dose-dependent manner. Notably, no morphological change was found until 6 h at any dose of silibinin (Fig. 1). These data suggest that silibinin inhibits macrophage activation in LPS-stimulated RAW 264.7 cells.

Inhibition of nitric oxide production by silibinin in LPS-stumulated macrophages

We investigated the effect of silibinin on NO production in LPS-stimulated RAW 264.7 cells since macrophages play a pivotal role in a host's defense against microbial infection through the production of variety of chemicals including NO (Hibbs *et al.*, 1987; Palmer *et al.*, 1988). LPS increased production of nitrite ≥20-fold over basal levels in RAW 264.7 cells (Fig. 2A). This induction of nitrite generation by LPS was inhibited by silibinin in a dose-dependent manner. No effect on cell viability was observed in any of the treatment groups and always exceeded 90% as determined by MTT assay (Fig. 2B).

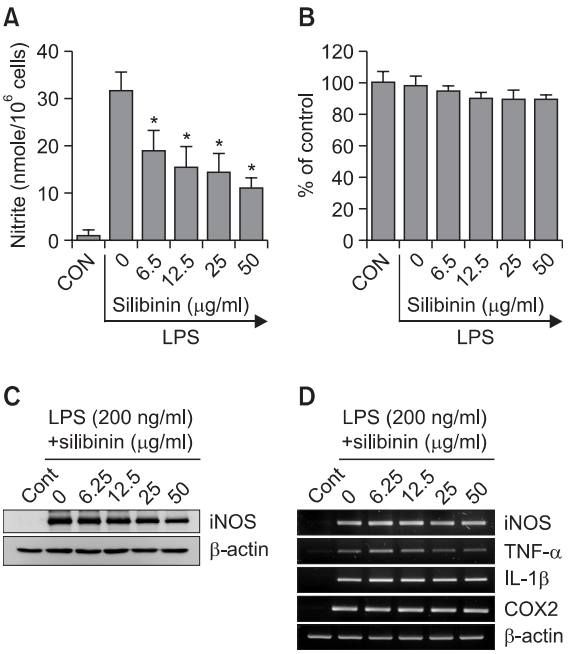

Fig. 2. Inhibition of iNOS expression by silibinin. (A, B) RAW 264.7 cells (5×10^5 cells/ml) were treated with the indicated concentrations of silibinin in the presence of LPS (200 ng/ml) for 18 h. (A) The supernatants were subsequently isolated and analyzed for nitrite. (B) Cells were subjected to MTT assay. (C, D) RAW 264.7 cells were treated with the indicated concentrations of silibinin in the presence of LPS for 18 h (C) or 8 h (D). (C) Expression of iNOS was analyzed by Western immunoblotting using an antibody specific for iNOS. (D) Total RNA was isolated and analyzed for mRNA expression levels of iNOS, TNF-α, IL-1β, and β-actin. Each column shows the mean ± S.D. of triplicate determinations. An *, indicates a response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $p<0.05$.

After RAW 264.7 cells were exposed to silibinin in the presence of LPS, the expression levels of iNOS gene were monitored by Western immunoblot analysis. As shown in Fig. 2C, iNOS protein production was inhibited by silibinin treatment. Control β-actin was constitutively expressed and was not affected by the treatment of silibinin. These results indicate that silibinin decreases the gene expression of iNOS, which is involved in inflammation (Hibbs *et al.*, 1987). RT-PCR analysis of iNOS, TNF-α, IL-1β and COX-2 mRNA of silibinin-treated macrophages showed no significant changes in transcription of these inflammatory mediators induced by LPS, although silibinin induced mild inhibition of iNOS and TNF-α mRNA production (Fig. 2D).

Inhibition of p38 MAPK phosphorylation by silibinin in LPS-stumulated macrophages

To further investigate the mechanism by which silibinin inhibits macrophage activation, we analyzed the effect of silibinin on MAPK signal transduction in LPS-stimulated macrophages. p38 MAPK phosphorylation is induced by LPS. In RAW 264.7 cells that were treated with silibinin in the presence of LPS for 30 min, the LPS-induced p38 MAPK phosphorylation was significanlty inhibited by silibinin (Fig. 3A). However, LPS-induced phosphorylation of ERK1/2 and JNK was not changed by silibinin.

Since we showed that p38 MAPK is a possible target for silibinin, we further investigated whether the p38 MAPK pathway is involved in LPS-induced macrophage activation. Therefore, we blocked the p38 MAPK pathway and monitored macrophage activation when RAW 264.7 cells were challenged with LPS. SB203580, a bicyclic imidazole compound, is a specific inhibitor of p38 MAPK (Cuenda *et al.*, 1995). PD98059 is a specific inhibitor of MEK-1, mitogen activated protein kinase/extracellular signal-regulated kinase 1, which is responsible for ERK1/2 activation (Dudley *et al.*, 1995). SB203580 inhibited LPS-induced nitrite generation, while PD98059 did not inhibit (Fig. 3B). These results suggest that silibinin inhibits p38 MAPK pathway, which is important in the regulation of

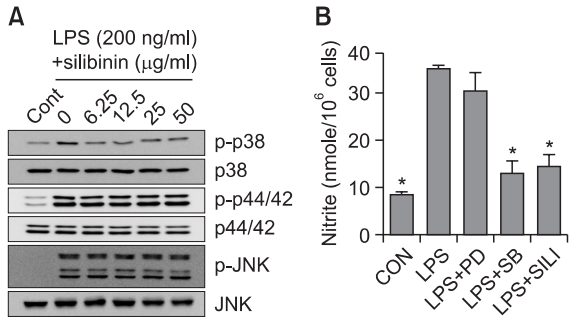

Fig. 3. Inhibition of p38 MAPK phosphorylation by silibinin in LPS-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were treated with silibinin for 30 min in the presence of LPS. The phosphorylation of p38 MAPK and ERK1/2 (p44/p42) was analyzed using Western blot assay. (B) RAW 264.7 cells were treated with PD98059 (50 μDM), SB203580 (30 μM), or silibinin (SILI, 50 μg/ml) for 18 h in the presence of LPS. The supernatants were subsequently isolated and analyzed for nitrite. Each column shows the mean ± S.D. of triplicate determinations. An *, indicates a response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $p<0.05$.

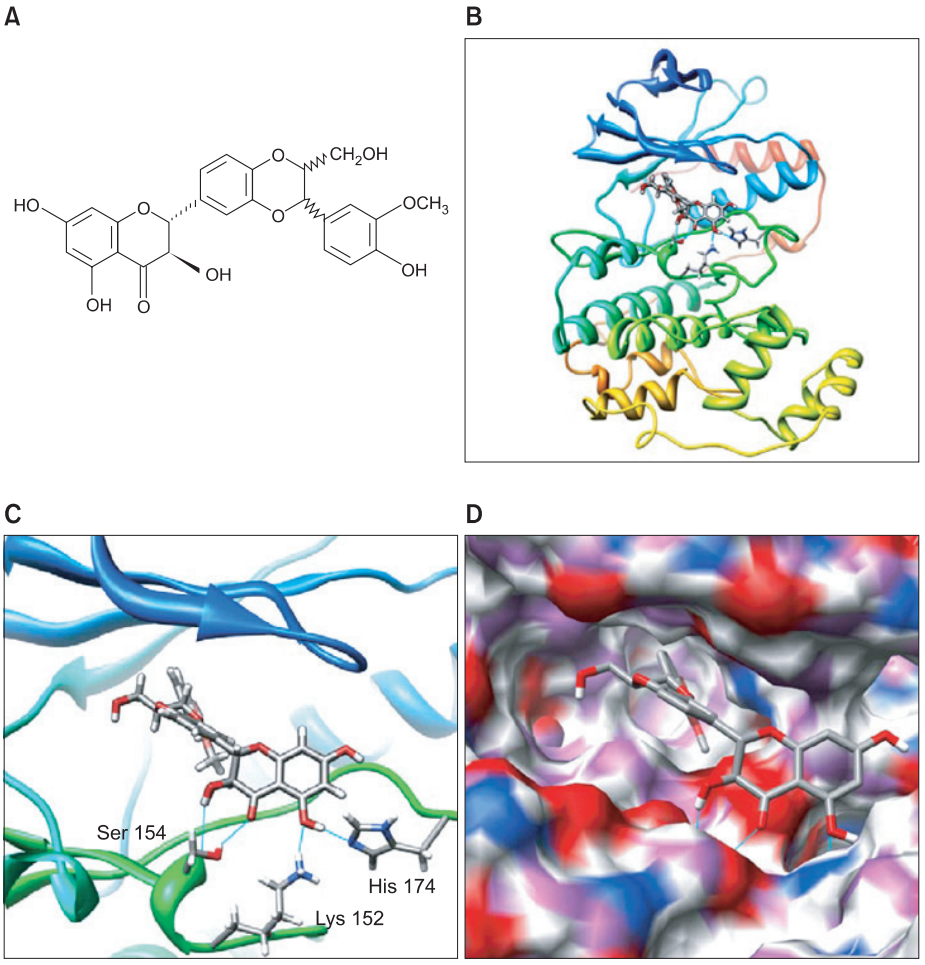

Fig. 4. Molecular Docking and Pose Generation. (A) Chemical structure of silibinin is shown. (B) A docking study was performed as described in Materials and methods. Silibinin was docked with p38 MAPK structure (PDB code: 1OUK). The protein residues are shown in a ribbon model. (C) The proposed binding pose of silibinin shows an interaction with Lys152, Ser154, and His174. (D) Space filling model showing the binding of silibninin in the ATP binding pocket of p38 MAPK.

macrophage activation by LPS.

Molecular docking

Because the activation of p38 MAPK, which plays an important role in macrophage activation, was inhibited by silibinin we performed a molecular docking of silibinin in silico to the ATP binding pocket of p38 MAPK using several protocols in the Schrödinger Suite of software. The chemical structure of silibinin, 2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-6-(3,5,7-trihydroxy-4-oxobenzopyran-2-yl) benzodioxin, is shown in Fig. 4A. By studying all the models returned, we found that silibinin formed some favorable connections and docked nicely within the p38 MAPK ATP binding site (Fig. 4B). Some important hydrogen bonds were formed between silibinin and both backbone and side chains of p38 MAPK including Lys152, Ser154, and His174 (Fig. 4C). A space filling model of p38 MAPK represented silibinin binding at the ATP binding pocket of p38 MAPK (Fig. 4D). These images were generated using the UCSF Chimera program (Pettersen *et al.*, 2004).

DISCUSSION

We demonstrate that silibinin treatment significantly attenuates LPS-induced macrophage activation by blocking p38 MAPK activation in the macrophage line RAW 264.7 cells. Our molecular modeling study suggests that silibilin interacts with amino acid residues (Lys152, Ser154, and His174) of p38 MAPK (1OUK) and occupies the ATP binding pocket of p38 MAPK. The p38 MAPK plays an important role in the LPS-induced signal pathway leading to the expression of a number of proinflammatory molecules (Lee and Young, 1996; Da Silva *et al.*, 1997; Chen and Wang, 1999). In the present study, we also showed that the p38 MAPK pathway is specifically involved in LPS-induced NO generation since NO production in the presence of p38 MAPK specific inhibitor, SB203580 was dramatically diminished. In contrast, PD98059, a specific inhibitor of MEK1 had no effect on NO production. This is further supported by our previous results showing the role of p38 MAPK in iNOS expression (Jeon *et al.*, 2000). The p38 MAPK also regulates LPS-induced production of cytokines including

TNF-α, IL-1, and IL-10 and TNF-induced IL-6 production in fibroblasts (Beyaert *et al.*, 1996; Foey *et al.*, 1998). Thus, silibinin, like SB203580, inhibits NO production and macrophage activation at least in part by blocking the p38 MAPK pathway. MAPKs have been suggested to be potential targets of silymarin and silibinin (Singh *et al.*, 2002; Kim *et al.*, 2009). Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of MAPKs and induction of apoptosis (Singh *et al.*, 2002). Silibinin block the activation of NF-κB, JNK, p38 MAPK, and ERK in osteoclast precursors in response to RANKL (Kim *et al.*, 2009).

Macrophages can be activated by cytokines or by microbial components (Stuehr and Nathan, 1989; Higuchi *et al.*, 1990), and play pivotal roles in both nonspecific and specific immunity. For nonspecific immunity, macrophages engulf and digest microorganisms and release inflammatory mediators such as NO. In specific immunity, macrophages act as antigen presenting cells and release cytokines such as TNF-α, IL-1, IL-6, and IL-12 to regulate helper T cells (Billack, 2006). Furthermore, activated macrophages suppress the growths of a variety of microbes and tumor cells (Lowenstein *et al.*, 1993). It was previously reported that silibinin has cancer-preventive and anticarcinogenic effects (Hogan *et al.*, 2007; Mokhtari *et al.*, 2008). Although macrophage activation and NO production are essential for proper defense mechanism, strong activation of macrophages causes the releases excessive cytokines and mediators, including NO, which has been implicated in many pathophysiological conditions including inflammation, atherosclerosis, and septic shock (Cohen, 2002). Immunomodulating compounds target macrophages due to the critical role that macrophage activation plays in innate immune response, (Li *et al.*, 2010; Vo *et al.*, 2012).

In summary, these experiments demonstrate that silibinin, a polyphenolic compound isolated from *Silybum marianum*, inhibits LPS-induced macrophage activation and NO generation. Based on our findings, the most likely mechanism that can account for this biological effect involves the negative regulation of the p38 MAPK pathway. Due to the critical role that macrophage activation plays in mediating inflammatory responses, the inhibitory effects of silibinin on macrophage activation suggest that silibinin may be a useful agent for inflammatory disease such as insulin-dependent *Diabetes mellitus*, rheumatoid arthritis, and sepsis.

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