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, acetalaminophen, 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
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 typhosa 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-fluorophenyl)-2-(4-methylsulphonylphenyl)-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% CO₂ 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⁻ 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 (Amerham 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 TG-3’, 5’-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 Å (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 (Petterson 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 (5×10⁵ 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.

| LPS (200 ng/ml) | silibinin (µg/ml) |
|----------------|------------------|
| CONTROL       | 0                |
| 6.25          | 12.5             |
| 25            | 50               |

Fig. 1. Inhibition of macrophage activation by silibinin. RAW 264.7 cells (5×10⁵ 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.
Inhibition of nitric oxide production by silibinin in LPS-stimulated 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). 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-stimulated 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 significantly 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 inflammation (Hibbs et al., 1987). RT-PCR analysis 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.
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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 (Petersen 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 silibinin 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 blocks 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 microbrial 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., 1983). It was previously reported that silibinin has cancer-inhibitory activity against a variety of microbes and tumor cells (Lowenstein et al., 2009). It was previously reported that silibinin has cancer-inhibitory activity against a variety of microbes and tumor cells (Lowenstein et al., 2009).

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 innate immune response, (Li et al., 2010; Vo et al., 2012).

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