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Silibinin Suppresses Mediators of Inflammation through the Inhibition of TLR4-TAK1 Pathway in LPS-induced RAW264.7 Cells

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Abstract Silibinin is the major bioactive compound of silymarin which is the mixture of flavonolignans extracted from milk thistle. Silibinin has been shown to possess anti-inflammatory activity. However, the underlying mechanisms still remain unclear. The aims of this study were to determine the effect of silibinin on molecular mechanism in lipopolysaccharide (LPS)-induced RAW264.7 macrophage cells. Here, we observed that silibinin attenuated the production of nitric oxide (NO) and its regulatory protein inducible nitric oxide synthase (iNOS) expression. The pro-inflammatory cytokine interleukin (IL)-1β was inhibited by silibinin in a time dependent manner. Moreover, silibinin decreased the expression of toll-like receptor (TLR)-4, TAK1, and IRF3. TLR-associated MAPK signaling pathway was also dramatically down-regulated in LPS-induced RAW 264.7 cells with presence of silibinin. Silibinin repressed oxidative stress-associated proteins including NOX4, G6PDH, and CuZnSOD, while silibinin increased the expression of GR and catalase in LPS-induced RAW264.7 cells. In the current study, silibinin suppresses the LPS-induced inflammation via modulation of TLR4-TAK1 signaling and subsequently attenuated the production of inflammation mediators in RAW264.7 cells. Therefore, we suggest that silibinin has a potential bioactivity for prevention and intervention of endotoxin-mediated inflammation and TLR4-TAK1-associated chronic diseases.

Keywords: *silibinin, TLR4, TAK1, MAPK, Inflammation, LPS, RAW264.7*

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1. Introduction

Host defense immune system plays a crucial role to help maintain homeostasis of the body. Innate immune response is caused when pattern recognition receptors such as Toll-like receptors (TLRs) sense the presence of infection via recognition of pathogen associated molecular patterns (PAMPs) [1]. Immune response is an essential physiological process in host defense system, while excessive immune reaction against to pathogen such as inflammation response can enhance to tissue damage, allergic symptom, endotoxemia, and metabolic diseases [2,3,4,5].

There are a number of pharmacological reagents have been developed to treat the inflammation since 18th centuries. These drugs are still linked to many adverse side effects including hepatic toxicity, malfunction of kidney, gastrointestinal injury, and immunosuppression [6,7,8,9]. Thus, it is necessary to search for reagent which can suppress inflammatory response without side effects.

Several studies involving in vitro and in vivo have shown that both edible and medical plant-isolated flavonoid and polyphenol act as a TLR4 signaling pathway suppressor in LPS-stimulated macrophage cell lines [10,11,12,13]. Silimaryin have been reported to inhibit the major pro-inflammatory cytokines by repressing the TNFα-mediated NF-κB translocation via inhibition of MAPK signaling [14]. Slimaryin, derived from the milk thistle *Silybum marinun*, is consist of three major constituents including isosilybins A, isosilybins B, and silibinin [15]. Silibinin is the most biologically effective component in silymarin, which prevent prostate cancer, hepatic damage, and cellular toxicity [16,17,18]. Moreover, it has been shown that silibinin repress lipopolysaccharide (LPS), okadaic acid, and ceramide-induced transcription factor nuclear factor-kappaB (NF-kB) pathway and inhibit the inflammation response in vitro and ex vivo [14,19]. NF-kB pathway is involved in LPS-TLR4 cascade and, leading to subsequent intracellular inflammatory response [20].

Silibinin seems to play an important role in pathogen-mediated inflammation. However, recent studies have uncovered a part of the molecular mechanism underlying whether silibinin regulates the expression of TLR4 signaling pathway in macrophage cell line. We therefore assessed whether silibinin regulate mediators of inflammation through the inhibition of TLR4 signaling cascade in LPS-stimulated RAW264.7 cells.

2. Materials and Methods

2.1. Reagents

Silibinin was purchased from Sigma (sc-202514; Santa Cruz, CA, USA). Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) was purchased from Sigma (St. Louis, MO, USA). Thiazolyl Blue tetrazolium bromide (MTT) was purchased from Alfa Aesar Chemical Inc. (Ward Hill, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin with streptomycin (PS) were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against to ERK, p-ERK, p38MAPK, p-p38MAPK, JNK, p-JNK, TAK1, p-TAK1, iNOS, TLR4, IRF3, p-IRF3, and IL-1β were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against to glutathione reductase (GR), catalase, NOX4, G6PDH, α-tubulin, and SOD1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Bio Rad (Hercules, CA, USA). Enhanced chemiluminescence system (ECL) was obtained from Thermo Fisher Scientific (San Jose, CA, USA).

2.2. Cell Culture and Viability

RAW264.7 cells were obtained from the American Type Culture Collection (ATCC) and were cultured in DMEM containing 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 100 μg/mL PS. Cells were grown at 37 °C in a 5 % CO_2/air environment. To evaluate the cell viability, RAW264.7 cells were plated at a concentration of 10,000 cells/well in a 96-well plate, and MTT assay was performed as described by Kim et al [21]. For the cell viability analysis, RAW264.7 cells were treated with 0, 7.5, 15, 30, 60, and 120 μM of silibinin.

2.3. Nitric Oxide Assay

RAW264.7 cells were seeded into six-well plates at a density of 1×10^5 cells per well. RAW264.7 cells were treated with sillibinin and were incubated for 1 h. LPS (1 μg/mL) was added to the culture; then, the cells were incubated for 24 h at 37 °C in a CO_2 incubator. Nitrite release in the culture media was determined using the Griess reaction and presumed to reflect the nitric oxide (NO) levels. After various treatments, 100 μL of cell culture medium was mixed with 100 μL Griess reagent and incubated at room temperature for 15 min. The NO concentration was determined at 540 nm using $NaNO_2$ as a standard.

2.4. Western Blot Analysis

RAW264.7 cells were washed in 1× phosphate-buffered saline (PBS), lysed in lysis buffer (1 M Tris-HCl, 0.5 M EDTA, 0.5 M EGTA, 1.5 M NaCl, 10 % NP-40, 10 % SDS, 10 % sodium deoxycholate, β-mercaptoethanol, 0.1 M PMSF, 0.1 M benzamidine, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 0.25 mg/mL pepstatin, 0.1 M sodium orthovanadate, phosphatase inhibitor cocktail 2, phosphatase inhibitor cocktail 3, 1 M sucrose, sodium fluoride, sodium pyrophosphate, and β-glycerol phosphate), and centrifuged to remove cell debris. Cytosolic and nuclear proteins were extracted using NE-PERTM nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manual. The protein content of the supernatant was determined using the Bradford assay. Protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Immune-Blot membrane). Membranes were immunoblotted with primary antibodies specific for iNOS, COX-2, p65, p-IκB-α, IκB-α, p-IKKα/β, IKKβ, p-ERK, ERK, p-p38, p38, p-JNK, JNK, and β-actin at 4°C overnight. Membranes were then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000) for 2 h. Bands were visualized using an ECL solution and LAS image software (Fuji, New York, NY, USA).

2.5. The Measurement of pH Values

RAW264.7 cells were incubated with silibinin for 24 h at CO_2 incubator, allowing the synchronization of silibinin binding onto cells. The supernatant were collected after incubation time from culture plate, respectively. Subsequently pH values were measured using a pH meter with micro-pH probe (Istek Co., Seoul, South Korea).

2.6. Statistical Analysis

All experiments were performed in triplicate. Differences among multiple groups were determined by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, using the SAS 9.0 software (SAS Institute, NC, USA). Values with different letters are significantly different, $p<0.05$.

3. Results

3.1. Silibinin Decreased the Nitric Oxide Production and the Expression of iNOS Protein in LPS-stimulated in RAW264.7 cells

To select the proper concentration of silibinin (Figure 1A) to conduct the evaluation of anti-inflammation in RAW264.7, we first investigated cell viability assay by using a MTT assay. As shown in Figure 1B, RAW264.7 cells were showed cytotoxicity at 120 μM. Thus, we selected 15, 30, and 60 μM of silibinin for the further experiments.

It has been known that LPS stimulates the production of NO in RAW264.7 cells. To test whether silibinin regulates the production of NO in RAW264.7 cells, we performed NO assay as described in Materials and Methods. Figure 2A showed silibinin suppressed the contents of NO in LPS-stimulated RAW264.7 cells in a dose dependent manner, while the production of NO were not observed when silibinin treated in RAW264.7 without LPS. The production of NO is a one of the final inflammatory response through the regulation of a several proteins including iNOS in RAW264.7 cells.

Next, western blot analysis has been conducted to evaluate the expression of iNOS protein in LPS-stimulated RAW264.7 cells. We found that silibinin consistently suppressed the expression of iNOS protein in LPS-stimulated RAW264.7 cells as shown in Figure 2B. In particular, LPS initiated the expression of iNOS at 12 h, and constantly increased until 18 h as shown in Figure 2C.

Silibinin dramatically repressed the expression of iNOS at 12 h and 18 h in LPS-stimulated RAW264.7 cells.

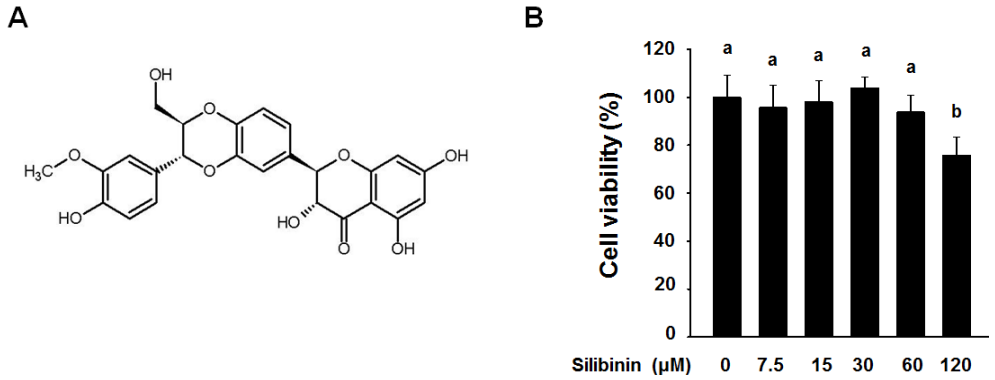

Figure 1. Effect of silibinin on the cell viability in RAW264.7 cells. (A) Chemical structure of silibinin. (B) Cell viability was determined using the MTT assay as described in the Materials and Methods

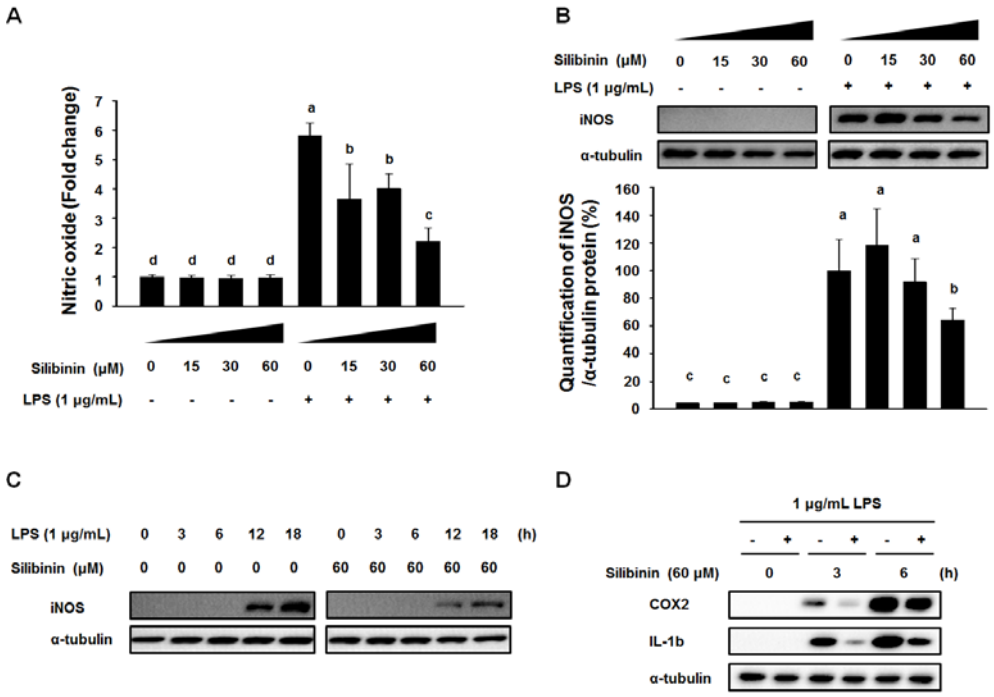

Figure 2. Silibinin suppresses the NO production through the regulation of iNOS, COX2, and IL-1β proteins. (A) The production of NO in LPS-induced RAW264.7 cells. (B) Western blot analysis of the iNOS in LPS-induced RAW264.7 treated with 0, 15, 30, and 60 κM of silibinin. (C) Time dependent analysis of iNOS protein in LPS-induced RAW 264.7 cells. (D) Western blot analysis of the pro-inflammatory cytokines including COX2 and IL-1β in LPS-induced RAW264.7 cells treated with silibinin

As shown in Figure 2D, western blot analysis also revealed that RAW264.7 cells treated with LPS were elevated the expression of COX2 and IL-1β proteins at 3 h and at 6 h, whereas silibinin dramatically decreased the LPS-induced protein expression of COX2 and IL-1β. Both of COX2 and IL-1β is downstream target of TLR4 signaling. Activated TLR4 signaling pathway leads to produce the inflammatory cytokine such as IL-1β, IL-6, and MCP-1 through regulation of pro-inflammatory cytokines genes in LPS-stimulated RAW264.7 cells [22]. These results prompt us to evaluate the expression of TLR4 signaling and its constituents in LPS-stimulated RAW264.7 with absence or presence of silibinin.

3.2. Effects of Silibinin on TLR4 Signaling Pathway in LPS-induced RAW264.7 Cells

The major constituent of TLR4 signaling pathway is TLR4, TAK1, and IRF3. To evaluate the effect of silibinin whether the modulation of TLR4 signaling pathway, we analyzed the expression of TLR4, TAK1, and IRF3 in LPS-stimulated RAW264.7 cells. Our results showed that TLR4 is continuously increased the protein expression from 0 h to 12 h in LPS-treated RAW264.7 cells and started to the degradation at 18 h as shown in Figure 3A. The expression of TLR4 protein was strongly decreased in LPS-stimulated RAW264.7 cells with presence of 60 μM silibinin.

Next, we examined the downstream target of TLR4 including TAK1 and IRF3 after LPS challenge in RAW264.7 cells. Western blot analysis showed that LPS caused the phosphorylation of TAK1 as shown in Figure 3B, while silibinin repressed the phosphorylation of TAK1 in LPS-stimulated RAW264.7 cells. We also observed that phosphorylation of IRF3 response to LPS was significantly decreased in RAW264.7 cells treated with silibinin.

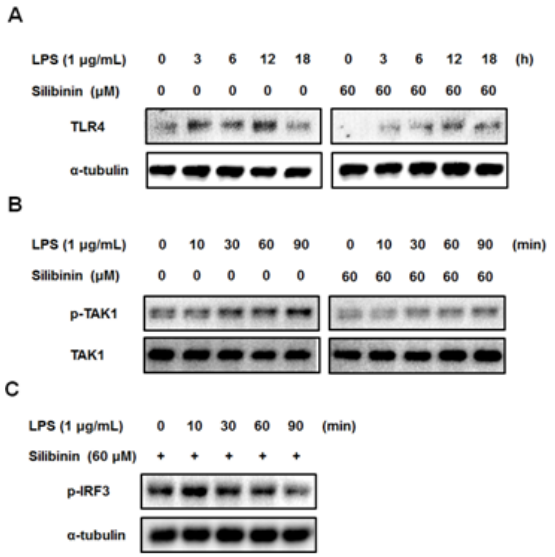

Figure 3. Silibinn decreases TLR4-TAK1expression response to LPS in RAW264.7 cells. (A) TLR4 was proved for antibody against to TLR4 and α-tubulin as a loading control. (B) Western blot were performed by incubating total lysates were with p-TAK1, TAK1, (C) p-IRF3, and α-tubulin specific antibodies

3.3. LPS-stimulated the Phosphorylation of p38 MAPK and JNK Were Suppressed in RAW264.7 Cells with Presence of Silibinin

TAK1 is a mitogen activated protein kinase (MAP3K) which is one of the constituent of MAPK signaling pathway [23]. To confirm the activity of p38MAPK, ERK, and JNK in RAW264.7 cells, western blot analysis has been performed. Kinetic analysis of protein expression showed that LPS strongly stimulated the phosphorylation of p38MAPK, JNK, and ERK in RAW264.7 cells with the presence of LPS at 30 min (Figure 4A). Silibinin strongly attenuated the LPS-stimulated the phosphorylation of p38MAPK and JNK in LPS-treated RAW264.7 cells. The ERK protein was showed a tendency to decrease the phosphorylation when silibinin treated in LPS-stimulated RAW264.7 cells. To clarify whether silibnin ameliorate the LPS-caused the phosphorylation of ERK, we treated a various concentration of silibinin (0, 7.5, 15, 30, and 60 μM) in LPS-stimulated RAW264.7 cells. As shown in Figure 4B, our data clearly showed that silibinin reduced the LPS-caused the phosphorylation of ERK.

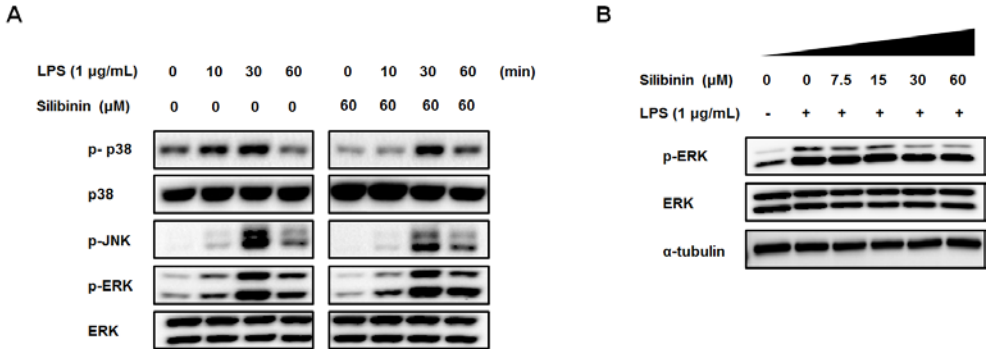

Figure 4. Silibinin inhibits the expression of MAPKs express in LPS-induced RAW264.7 cells. (A) RAW264.7 cells were pre-treated with indicated concentration of silibinin for 4 hours, and then co-treat with LPS for 30 min. Western blot were conducted by incubating total lysate were with p-p38MAPK, p38MAPK, p-ERK, ERK, p-JNK, and JNK specific antibodies. (B) p-ERK protein was proved for antibodies against to p-ERK and ERK as a loading control

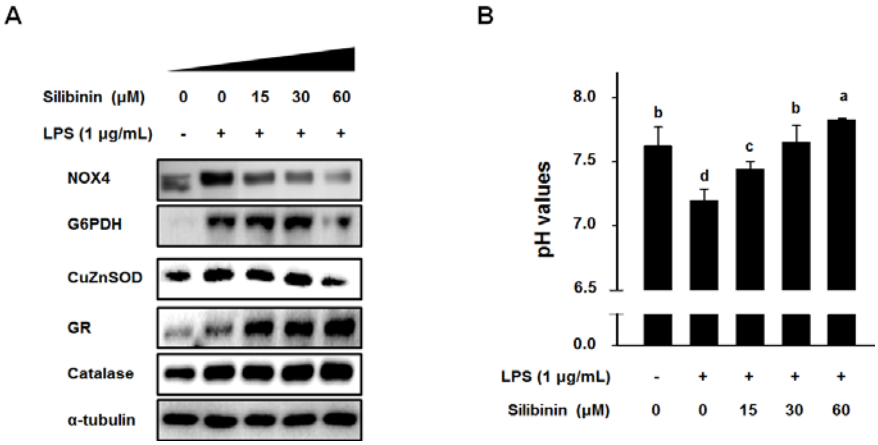

Figure 5. Silibinin regulated inflammatory stimuli-induced oxidative stress-associated proteins and inflammation-medicated low pH values in RAW264.7 cells. (A) Western blot were performed by incubating total lysates were with NOX4, G6PDH, CuZnSOD, GR, catalase, and α-tubulin specific antibodies. (B) Evaluation of pH values in LPS-induced RAW264.7 cells.

3.4. Silibinin Regulated the Oxidative Stress-Associated Enzymes and Repressed LPS-induced pH Changes in RAW264.7 Cells

It has been known that activated TLR4 signaling also initiates the oxidative stress response in macrophage cells [24]. Thus, we assessed the expression of oxidative stress regulatory proteins (NOX4, G6PDH, CuZnSOD, GR, and catalase) in LPS-stimulated RAW264.7 cells with absence

or presence of silibinin. The expression of NOX4 and G6PDH protein were increased in LPS-stimulated RAW264.7 cells, whereas silibinin exhibited a significantly reduced the expression of NOX4 and G6PFDH proteins compared with RAW264.7 cells treated with LPS. In addition, silibinin treatment in LPS-stimulated RAW264.7 cells elevated the GR and catalase expression in a dose dependent manner compared with the presence of single LPS-treated RAW264.7 cells (Figure 5A).

In general, oxidative stress induce low environmental pH around macrophages and aggravate the inflammatory response [25]. We next analyzed the effect of silibinin on pH values in LPS-stimulated RAW264.7 cells. As shown in Figure 5B, we observed that LPS promoted a decreased the media pH, while silibinin significantly suppressed LPS-mediated pH alteration in growth media.

4. Discussion

Recent study has suggested silibinin has a potent effect to prevent neuro-inflammation in ischemic brain through suppressing NF-κB signaling [26]. Moreover, silibinin have been shown to alleviate TPA-mediated COX2 expression in breast cancer cell lines [27] and to inhibit lung cancer progression through markedly decrease in COX2 protein and subsequently targeting NF-κB signaling [28]. NF-κB signaling is well known to trigger the production of pro-inflammatory cytokines and is regulated by COX2 [29]. Aforementioned studies potentially suggest that silibinin may inhibit inflammation response through the regulation of TLR4 signaling, which is upstream pathway of NF-κB signaling, in macrophage. The underlying mechanisms of the anti-inflammatory effect whether silibinin regulates TLR4 signaling, however, have not been previously reported.

The mediators of inflammation NO, IL-6, and IL-1β are caused by expression of COX2 and iNOS protein, respectively, as part of the inflammation response [30,31]. Suppression of NO and inflammatory cytokines (IL-6 and IL-1β) have been used as a therapeutic target for the prevention and intervention of inflammation-associated diseases [10,32,33,34]. The pharmacological reagent against to inflammation has been accordingly developed negatively regulating the transcriptional factor of pro-inflammatory constituents [35,36,37]. The RANKL-induced DNA binding activity of NFATc1 and its modulators NF-κB and AP-1 were decreased by silibinin osteoclast differentiated RAW264.7 cells. The expression of NF-κB signaling following LPS stimulation is mediated by the TLR4 signaling, which is also associated to the transcriptional regulation of pro-inflammatory cytokines. Hence, we investigated whether silibinin could regulate the mediators of inflammation via TLR4 signaling cascade in LPS-stimulated RAW264.7 cells.

In this study, we observed that silibinin effectively inhibited of LPS induced NO production through the discouragement of expression both iNOS and COX2 proteins, respectively. We also found that silibinin suppressed the IL-1β expression following LPS stimulation in RAW264.7 cells. Thus, our results indicate that silibinin represses mediators of inflammation such as NO production by targeting iNOS and COX2 proteins.

LPS stimulation is closely associated with TLR4 dependent signaling, which is a crucial signal transduction pathway in pathogen-mediated inflammation. In present study, we assessed whether silibinin might affect TLR4 signaling cascade, the upstream signaling of iNOS and COX2, in LPS-induced RAW264.7 cells. Silibinin inhibited the phosphorylation of TAK1 by suppressing TLR4 protein, which subsequently prevented downstream of TLR4 signaling cascade. These results are similar to those found for anti-inflammatory pharmaceutical compound such as glucocorticoids [38], which repress the LPS induced TLR4-TAK1 signaling in macrophage cells.

In previous, TAK1 has been shown an essential kinase in inflammatory signaling [39]. In particular, TAK1 antagonist inhibits advanced glycation end products-induced inflammatory response to discourage mediators of inflammation via MAPKs and NF-κB signaling pathways such as those involving p38MAPK, JNK, and IKK [40]. In this study, we found that silibinin could inhibit TLR4 signaling. This result was demonstrated by the attenuated expression of TLR4 protein and phosphorylation of TAK1. Phosphorylation of the p38MAPK and JNK is an important reaction to the production of pro-inflammatory cytokine. We also observed that the production of pro-inflammatory cytokines suppressed by the MAPKs in LPS-stimulated RAW264.7 cells.

LPS-stimulated RAW264.7 cells could cause oxidative stress, leading to disrupted activities of endogenous antioxidants [41]. Significantly elevated GR and catalase activities suggest a critical role for this enzyme in cell and tissue protection against the oxidative stress [42]. Therefore, the increase in GR and catalase protein expression in the current study suggests that silibinin is capable of suppressing LPS-induced oxidative stress in RAW267.7 cells. These findings are partially similar to a few other reports [43,44]. Finally, to clarify the physiological relevance of our in vitro results demonstrating the inflammation-induced somatic low pH value [45,46,47], we assessed the levels of pH value in LPS-stimulated RAW264.7 cells. We observed that silibinin dramatically ameliorated the LPS-caused low pH values.

In conclusion, our data provides a part of evidence indicating that silibinin is a potential natural bioactive compound that leads to repress the TLR4-TAK1 signaling cascade, and subsequently inhibiting the mediators of inflammation in LPS-induced RAW264.7 cells. Hence, silibinin might be a useful functional food ingredient to attenuate the extracellular pathogen-mediated inflammation and TLR4-TAK1 signaling-associated chronic diseases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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