

Protection against lipopolysaccharide-induced sepsis and inhibition of interleukin-1 β and prostaglandin E2 synthesis by silymarin

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Abstract

Silymarin is known to have hepatoprotective and anticarcinogenic effects. Recently, anti-inflammatory effect of silymarin is attracting an increasing attention, but the mechanism of this effect is not fully understood. Here, we report that silymarin protected mice against lipopolysaccharide (LPS)-induced sepsis. In this model of sepsis, silymarin improved the rate of survival of LPS-treated mice from 6 to 38%. To further investigate the mechanism responsible for anti-septic effect of silymarin, we examined the inhibitory effect of silymarin on interleukin-1 β (IL-1 β) and prostaglandin E2 (PGE2) production in macrophages. Silymarin dose-dependently suppressed the LPS-induced production of IL-1 β and PGE2 in isolated mouse peritoneal macrophages and RAW 264.7 cells. Consistent with these results, the mRNA expression of IL-1 β and cyclooxygenase-2 was also completely blocked by silymarin in LPS-stimulated RAW 264.7 cells. Moreover, the LPS-induced DNA binding activity of nuclear factor- κ B/Rel was also inhibited by silymarin in RAW 264.7 cells. Taken together, these results demonstrate that silymarin has a protective effect against endotoxin-induced sepsis, and suggest that this is mediated, at least in part, by the inhibitory effect of silymarin on the production of IL-1 β and PGE2.

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1. Introduction

Silymarin is a polyphenolic flavonoid antioxidant isolated from the fruits and seeds of the milk thistle, *Silybum marianum* [1]. It is well known that silymarin protects against hepatotoxicity caused by a variety of agents [2,3]. Various studies also indicate that silymarin exhibits cancer-preventive and anticarcinogenic effects [4,5]. Moreover, silymarin possesses a number of additional biological effects, such as an antioxidative activity [6], an anti-inflammatory effect [7], and an inhibitory action on tumor necrosis factor- α (TNF- α) expression and ornithine decarboxylase activity [8,9].

Sepsis is a systemic response to infection, and septic shock develops in a number of patients after surgery as a complication [10,11]. The most common cause of sepsis is an exposure to the structural component of a Gram-negative bacterial membrane, LPS, and key symptoms include hypotension and vasoplegia, which may lead to the multiple organ dysfunction and ultimately death [10,12]. Bacterial LPS in the bloodstream induces the overproduction of various inflammatory mediators, such as IL-1 β , TNF- α , nitric oxide, and PGE2, and large amount of inflammatory mediators produced in the body are thought to contribute to the LPS-induced symptoms of septic shock and mortality [13].

IL-1 β is a cytokine involved in inflammatory and immunological processes and produced by a variety of cell types, such as monocytes/macrophages, fibroblasts and endothelial cells [14]. It is well known that IL-1 β contributes to the development of sepsis and induces the production of inflammatory mediators and other cytokines involved in sepsis [15]. Fisher *et al.* reported that the mortality of

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Abbreviations: COX-2, cyclooxygenase-2; IL-1 β , interleukin-1 β ; LPS, lipopolysaccharide; NF- κ B/Rel, nuclear factor κ B/Rel; PGE2, prostaglandin E2; RT-PCR, reverse transcription-polymerase chain reaction; TNF- α , tumor necrosis factor- α .

patients with sepsis is reduced by blocking the activity of IL-1 β by IL-1 receptor antagonists. During inflammation and sepsis, many IL-1 β -induced changes are mediated by prostaglandins, particularly PGE2 that is produced by cyclooxygenase-2 (COX-2) from arachidonic acid and is also known to be involved in sepsis. Moreover, toxicity after the administration of a lethal dose of LPS can be decreased by treatment with nonsteroidal anti-inflammatory drugs, such as indomethacin or ibuprofen [16], which is known to be a COX-2 inhibitor, and accumulating data suggest that the inhibition of COX-2 is actually a key mechanism whereby nonsteroidal anti-inflammatory drugs diminish inflammation [17]. Therefore, these reports suggest that IL-1 β and PGE2 are important inflammatory mediators of sepsis.

The objective of the present study was to investigate whether silymarin can block the LPS-induced sepsis and the gene expression of inflammatory mediators, such as IL-1 β and PGE2, involved in sepsis. Our results will give an insight into the mechanism of the anti-inflammatory effect of silymarin.

2. Materials and methods

2.1. Chemicals, animals, and cell culture

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Silymarin was dissolved in DMSO and freshly diluted in culture media for all *in vitro* experiments. Virus-free female BALB/c mice were purchased from Dae Han Laboratory Animal Research Center Co., Ltd. and cared for as described previously [18]. For *in vivo* administration, silymarin was dissolved in a water-based dosing solution containing 0.9% sodium chloride (w/v), 3% ethanol (v/v), 1% Tween-80 (v/v), and 6.6 mM of sodium hydroxide as described previously [19]. RAW 264.7 cells (ATCC TIB71) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37° in 5% CO₂ humidified air. Peritoneal macrophages were harvested by sterile peritoneal lavage using PBS, washed, resuspended in culture medium, and plated at 2 × 10⁶ cells/mL. Nonadherent cells were removed by repeated washing after 2-hr incubation at 37°.

2.2. Survival study

The BALB/c mice used in this experiment were 6 weeks of age. The mice were divided into three groups, and silymarin and vehicle (water based-dosing solution as described previously) was administered orally at 50 mg/kg 2 and 0 hr before LPS administration. LPS (from *Salmonella typhosa*) was administered intraperitoneally (i.p.) at a dose of 15 mg/kg, and survival of mice was monitored every 6 hr for 60 hr.

2.3. Measurement of IL-1 β and PGE2 secretion

Mouse peritoneal macrophages and RAW 264.7 cells were plated at 2 × 10⁶ and 5 × 10⁵ cells/mL, respectively, and stimulated with LPS (200 ng/mL) in the presence or absence of silymarin (6.25, 12.5, 25, or 50 μ g/mL) for 24 hr. Culture supernatants of culture medium were collected and the concentration of IL-1 β and PGE2 was determined using a TiterZyme-EIA kit and Prostaglandin E2 EIA system, respectively, according to the manufacturer's instructions.

2.4. Reverse transcription–polymerase chain reaction (RT–PCR)

The expressions of the mRNA transcripts of IL-1 β (forward primer: 5'-TGCAGAGTTCCTCCCAACTGGTACATC-3', reverse primer: 5'-GTGCTGCCTAATGTCCCCTTGAATC-3'), COX-2 (forward primer: 5'-TTTGTGAGTCA-TTCACCAGACAGAT-3', reverse primer: 5'-CAGTATT-GAGGAGAACAGATGGGATT-3'), and β -actin (forward primer: 5'-TGGAAATCCTGTGGCATCCATGAAAC-3', reverse primer: 5'-TAAAACGCAGCTCAGTAACAGTC-CG-3') were evaluated by RT–PCR as described previously with slight modifications [20]. Briefly, total RNA was isolated using Tri Reagent as described previously [21]. Equal amounts of RNA were reverse transcribed into cDNA using oligo(dT)₁₅ primers. Samples were heated to 94° for 5 min and cycled 25 times at 94° for 30 s, and 55, 60, and 60° (for IL-1 β , COX-2, and β -actin, respectively) for 30 s, and 72° for 45 s, and this was followed by an additional extension step at 72° for 5 min. PCR products were electrophoresed in a 1.5% agarose gel and followed by ethidium bromide staining and photography.

2.5. Electrophoretic mobility shift assay

Nuclear extracts were prepared as described previously [20]. The protein content of the nuclear extracts was determined using a Bio-Rad protein assay kit according to the manufacturer's instructions. The oligonucleotide sequence for nuclear factor κ B/Rel (NF- κ B/Rel) was 5'-GATCTCAGAGGGGACTTTCCGAGAGA-3' [22]. Double-stranded oligonucleotides were end-labeled with [γ -³²P]ATP. Nuclear extracts (5 μ g) were incubated with 2 μ g of poly(dI-dC) and a ³²P-labeled DNA probe, and DNA binding activity was analyzed using a 4.8% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography. The specificity of binding was examined by competition with an unlabeled oligonucleotide.

2.6. Statistical analysis

The mean \pm SD was determined for each treatment group in each experiment. Significance was determined

by either Dunnett's two-tailed *t*-test for comparison between two groups or by ANOVA, followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Protective effect of silymarin on LPS-induced sepsis in mice

Because silymarin is known to have anti-inflammatory activity, we tested the effect of silymarin on LPS-induced sepsis. The administration of a high dose of LPS (15 mg/kg, i.p.) to conscious mice resulted in a survival rate of 5% after 48 hr. However, the survival rate was increased to 33% in mice treated with silymarin (50 mg/kg 2 and 0 hr before LPS treatment, oral administration) (Fig. 1). The increase of survival rate of mice treated with silymarin was observed after 30 hr, and survival rate of silymarin-treated group was statistically different from LPS-treated control group ($P < 0.05$, Dunnett's test).

3.2. Inhibition of IL-1 β and PGE2 production in LPS-stimulated macrophages

To investigate the effect of silymarin on IL-1 β and PGE2 production, we measured the accumulation of IL-1 β and PGE2 in culture media. In mouse peritoneal macrophages, treatment of LPS caused 5.2- and 6-fold induction of IL-1 β and PGE2 production. Silymarin was

found to dose dependently suppress the LPS-induced production of IL-1 β and PGE2 in isolated mouse peritoneal macrophages, and 50 μ g/mL silymarin caused 84 and 91% inhibition, respectively ($P < 0.05$, Dunnett's test) (Fig. 2). To further confirm, we also examined the effect of silymarin on IL-1 β and PGE2 production in a mouse macrophage cell line, RAW 264.7 stimulated with LPS. Figure 3 shows that the increased production of IL-1 β and PGE2 in LPS-stimulated RAW 264.7 cells was also inhibited by silymarin treatment in a dose-related manner. Treatment of high dose of silymarin (50 μ g/mL) caused 74 and 94% inhibition of the production of IL-1 β and PGE2, respectively ($P < 0.05$, Dunnett's test). The concentration and duration of silymarin treatment used in these studies had no significant effect on the viability of isolated mouse peritoneal macrophages and RAW 264.7 cells (data not shown).

3.3. Inhibition of IL-1 β and COX-2 mRNA expression in LPS-stimulated RAW 264.7 cells

The effect of silymarin on IL-1 β and COX-2 mRNA expression was examined by RT-PCR. As shown in Fig. 3, the expression of IL-1 β and COX-2 mRNAs was not detected in unstimulated RAW 264.7 cells, but RAW 264.7 cells expressed a high level of IL-1 β and COX-2 mRNAs when stimulated with LPS (200 ng/mL) for 12 hr. Furthermore, silymarin suppressed the LPS-stimulated IL-1 β and COX-2 mRNA expression in a dose-dependent manner (Fig. 4). In contrast, the level of β -actin mRNA remained the same under these conditions.

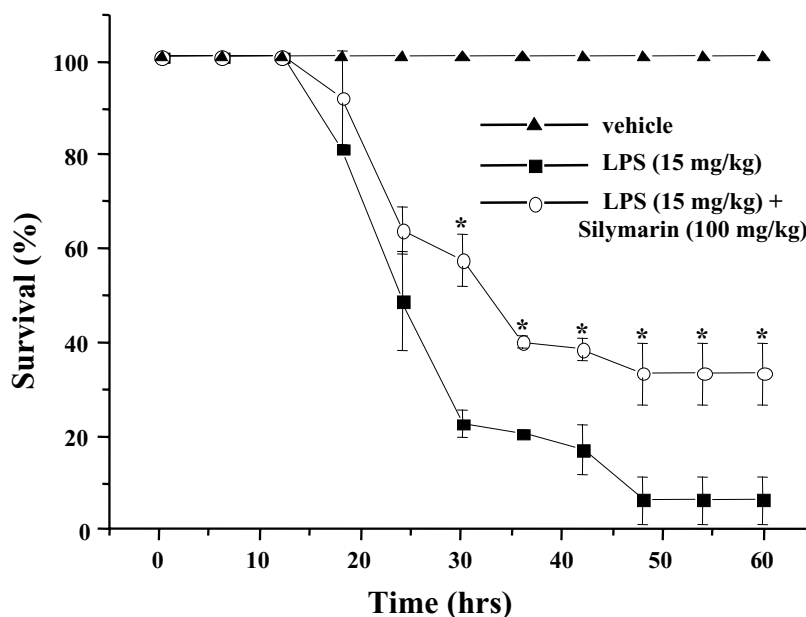


Fig. 1. Effect of silymarin on survival rate of mice treated with LPS (15 mg/kg). Different groups (N = 15) of animals were treated with vehicle (▲, DMSO), vehicle plus LPS (■, 15 mg/kg, i.p.), or LPS plus silymarin (○, 50 mg/kg at 2 and 0 hr before LPS treatment). The survival rate was measured every 6 hr throughout this experiment. Each column shows the mean \pm SD of triplicate determinations. (*) Response that is significantly different from the control group as determined by Dunnett's two-tailed *t*-test at $P < 0.05$.

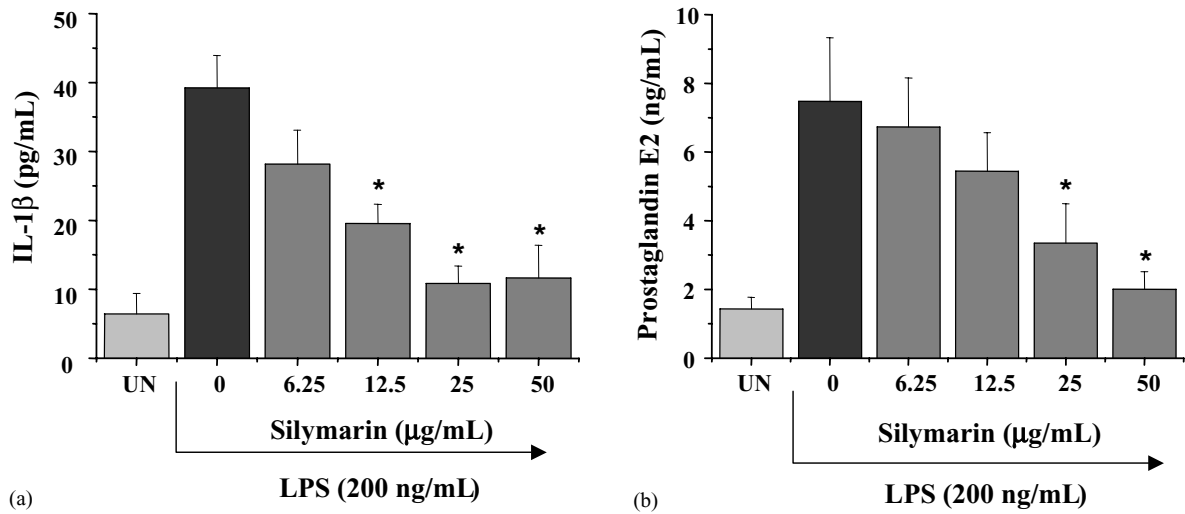


Fig. 2. Inhibition of IL-1 β and PGE2 production by silymarin in LPS-stimulated mouse peritoneal macrophages. Peritoneal adherent cells were pretreated with the indicated concentrations of silymarin for 1 hr before being incubated with LPS (200 ng/mL) for 24 hr. The culture supernatants were subsequently collected and analyzed for IL-1 β (a) and PGE2 (b) production as described in Section 2. Each column shows the mean \pm SD of triplicate determinations. (*) Response that is significantly different from the control group as determined by Dunnett's two-tailed *t*-test at $P < 0.05$.

3.4. Suppression of LPS-induced NF- κ B/Rel DNA binding activity by silymarin in RAW 264.7 cells

It is well known that NF- κ B/Rel is involved in the regulation of various immune and inflammatory responses, and that it is also an important transcriptional regulator of IL-1 β and COX-2 genes [23,24]. Moreover, a couple of previous studies have shown that silymarin has an inhibitory effect on NF- κ B/Rel activity in HepG2 (human hepatoblastoma-derived cell line) and U937 (human histiocytic lymphoma) cells [7,25]. Therefore, to investigate whether the transcription factor NF- κ B/Rel is a target of silymarin in RAW 264.7 cells, we performed an electrophoretic mobility shift assay. Treatment of RAW 264.7 cells with

200 ng/mL of LPS caused an increase in the DNA binding activity of NF- κ B/Rel transcription factor within 30 min (Fig. 5). However, silymarin markedly suppressed this LPS-induced NF- κ B/Rel DNA binding in a concentration-dependent manner.

4. Discussion

In the present study, we clearly demonstrated that silymarin inhibits the LPS-induced production of IL-1 β and PGE2, and that it protects mice against LPS-induced sepsis. It is well known that both IL-1 β and PGE2 play a crucial role in the development of LPS-induced sepsis

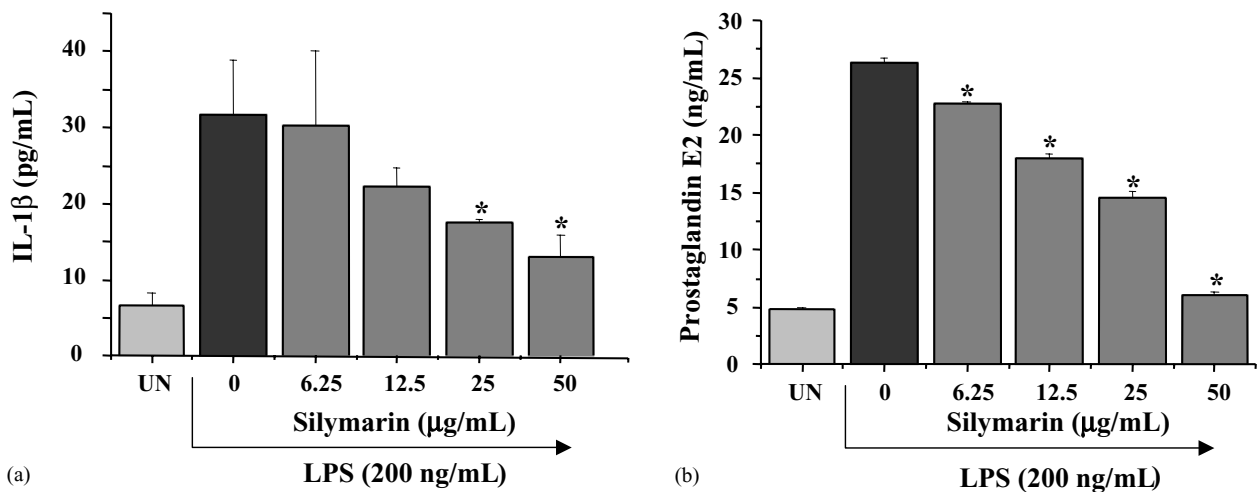


Fig. 3. Inhibition of IL-1 β and PGE2 production by silymarin in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of silymarin for 1 hr before being incubated with LPS (200 ng/mL) for 24 hr. The culture supernatants were subsequently collected and analyzed for IL-1 β (a) and PGE2 (b) production as described in Section 2. Each column shows the mean \pm SD of triplicate determinations. (*) Response that is significantly different from the control group as determined by Dunnett's two-tailed *t*-test at $P < 0.05$.

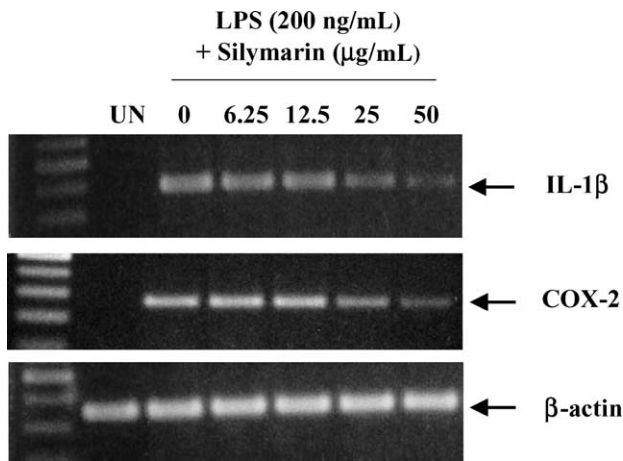


Fig. 4. Inhibition of IL-1 β and COX-2 mRNA expression by silymarin in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with silymarin (6.25, 12.5, 25 or 50 μ g/mL) for 1 hr before being incubated with LPS (200 ng/mL) for 12 hr. Total RNA was isolated, and IL-1 β and COX-2 mRNA expression was determined by RT-PCR. One of two representative experiments is shown.

[15,17]. IL-1 β is produced during inflammation, injury, or infection, and induces the expression of a variety of genes. The synergistic action of IL-1 β and TNF- α in the development of sepsis is also well known [26], but IL-1 β alone reproduces the systemic and pathological effects of sepsis in animals and human [27,28], and pretreatment of IL-1 receptor antagonists reduced the mortality of patients with sepsis [29]. Moreover, Ando *et al.* also showed that cerivastatin improved the survival of mice with

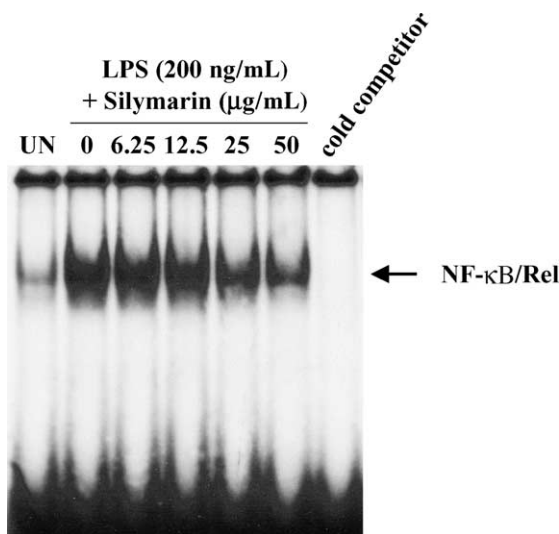


Fig. 5. Inhibition of NF- κ B/Rel DNA binding activity by silymarin in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of silymarin for 1 hr before being incubated with LPS (200 ng/mL) for 30 min. Nuclear extracts were then prepared, and NF- κ B/Rel DNA binding was determined by electrophoretic mobility shift assay. The binding specificity was determined using the unlabeled wild-type probe (100-fold in excess) to compete with the labeled oligonucleotide. The result presented is a representative of three independent experiments.

LPS-induced sepsis by inhibiting the production of IL-1 β . These reports show that IL-1 β is important for the development of sepsis. In the present study, we assessed the effect of silymarin on IL-1 β production to elucidate the possible mechanism responsible for the anti-septic effect of silymarin. Our results show that silymarin has an inhibitory effect on the secretion of IL-1 β and the expression of IL-1 β gene in mouse peritoneal macrophages and RAW 264.7 cells. PGE2 is another key mediator, which is involved in inflammation and sepsis, and has an important role in the regulation of fever and pain. Also, many IL-1 β and TNF- α -induced changes are mediated by PGE2. Moreover, it has been reported that increases in PGE2 are related with a pain during inflammation evoked by IL-1 β and TNF- α , and IL-1 β also lowers the threshold of pain primarily by increasing PGE2 synthesis [30]. Accordingly, it has been intensively investigated to understand how PGE2 production and COX-2 expression are involved in inflammatory disease and infection. PGE2 has also been found to have a crucial role in the pathogenesis of sepsis [31,32]. As was found for IL-1 β , treatment with PGE2 receptor antagonist restored alterations caused by burn sepsis [33]. Shoup and co-workers also reported that the COX-2 inhibitor NS-398 improved survival after burn infection. These reports suggest that sepsis can be reversed by inhibiting PGE2 production. Therefore, we investigated the effect of silymarin on the production of PGE2. In this report, we demonstrated the inhibitory effect of silymarin on PGE2 production and COX-2 expression in LPS-stimulated macrophages.

Of the various transcriptional regulatory factors involved in immunoregulatory gene expression, NF- κ B/Rel is the most important in terms of directing the transcription of many inflammatory genes after exposure to bacterial LPS [34]. Many stimuli, including LPS, cytokines, oxidants and viruses, activate NF- κ B/Rel *via* several signal transduction pathways. In turn, the activation of NF- κ B/Rel transcription factor leads to the increased production of various inflammatory mediators, including IL-1 β , TNF- α , NO, and PGE2, and adhesion molecules, such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin [23,24,35]. The development of sepsis is also known to be dependent on NF- κ B/Rel, and Arnalich and co-workers reported that NF- κ B/Rel activity in the peripheral blood mononuclear cells of patients with severe sepsis is strongly correlated with the severity of illness. The importance of NF- κ B/Rel in the expression of inflammatory cytokines may explain this effect. To investigate the mechanism of inhibitory effect of silymarin on IL-1 β and PGE2 production, we tested the effect of silymarin on LPS-induced NF- κ B/Rel DNA binding activity. In the present study, we demonstrate that LPS-induced NF- κ B/Rel DNA binding is completely blocked by silymarin treatment in RAW 264.7 cells. Taken together, our results suggest that the inhibition of the LPS-induced production of IL-1 β and PGE2 by silymarin

is mediated, at least in part, by the inhibition of the NF- κ B/Rel transcription factor.

In summary, this study demonstrated that silymarin has an anti-septic effect, and this was, at least in part, mediated by the inhibitory action of silymarin on the production of IL-1 β and PGE₂. The results of this study suggest that silymarin may be considered as a possible therapeutic agent for a variety of acute inflammatory diseases.

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