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Anti-inflammatory effects and corresponding mechanisms of cirsimaritin extracted from *Cirsium japonicum* var. *maackii* Maxim

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ABSTRACT

In this study, we investigated the anti-inflammatory effects and mechanisms of cirsimaritin isolated from an ethanol extract of the aerial parts of *Cirsium japonicum* var. *maackii* Maxim. using RAW264.7 cells. The extract and its flavonoid cirsimaritin inhibited nitric oxide (NO) production and inducible nitric oxide synthase expression in RAW264.7 cells. Cirsimaritin inhibited interleukin-6, tumor necrosis factor-α, and NO production in a concentration-dependent manner in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. From a western blot study, pretreatment with cirsimaritin inhibited phosphorylation/degradation of IκBα and phosphorylation of Akt in LPS-stimulated RAW264.7 cells. Moreover, cirsimaritin suppressed activation of LPS-induced transcription factors, such as c-fos and signal transducer and activator of transcription 3 (STAT3), in RAW264.7 cells. Collectively, these results show that cirsimaritin possesses anti-inflammatory activity, which is regulated by inhibition of c-fos and STAT3 phosphorylation in RAW264.7 cells.

Cirsium japonicum is a medicinal plant and a perennial herbaceous species that is found in mountains and fields in Korea, China, and Japan. The medicinal benefits of *C. japonicum* have been reported as antidiabetic, anti-tumor, antioxidant, anti-inflammatory, and antifungal effects.[1] The major components of *C. japonicum* have been characterized as flavonoids,[2] triterpenes,[3] wax,[4] polyolefins, and some acetylenes.[5] Among these components, the flavonoids, comprised of cirsimaritin,[6] pectolinarin,[7] 5,7-dihydroxy-6,4′-dimethoxyflavone, and hispidulin-7-neohesperidoside,[8] have been studied for their medicinal activities.

The anti-inflammatory properties of extracts from various plants have been investigated for several decades.[9] Inflammation is the first biological response of the immune system to infection or irritation. Oxidative stress is an important etiological and/or triggering factor for inflammatory disease.[10] The damaging effects of oxidative stress are well known in the inflammatory process.[11] Defective responses in both the innate and the adaptive immune systems in inflammatory disease have been reported.[12] In patients with inflammatory disease, the characteristics of cells mediating innate immunity, such as neutrophils, macrophages, dendritic cells, and natural killer cells, are altered; an abnormal mucosal T helper (Th) cell response and overexpression of cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-12, and IL-6, were also found.[13] During normal physiology, nitric oxide (NO) is synthesized from L-arginine with the involvement of constitutive NO synthases, such as eNOS and nNOS, whereas high levels of NO synthases, especially inducible nitric oxide synthase (iNOS), are indicative of inflammatory disease.[14]

Polyphenolic compounds play an important role in adsorbing and neutralizing free radicals, and quenching singlet and triplet oxygen. They also have metal-chelating potential and the ability to disrupt oxidizing chain reactions.[15] However, the molecular mechanisms of the anti-inflammatory activities of *C. japonicum*

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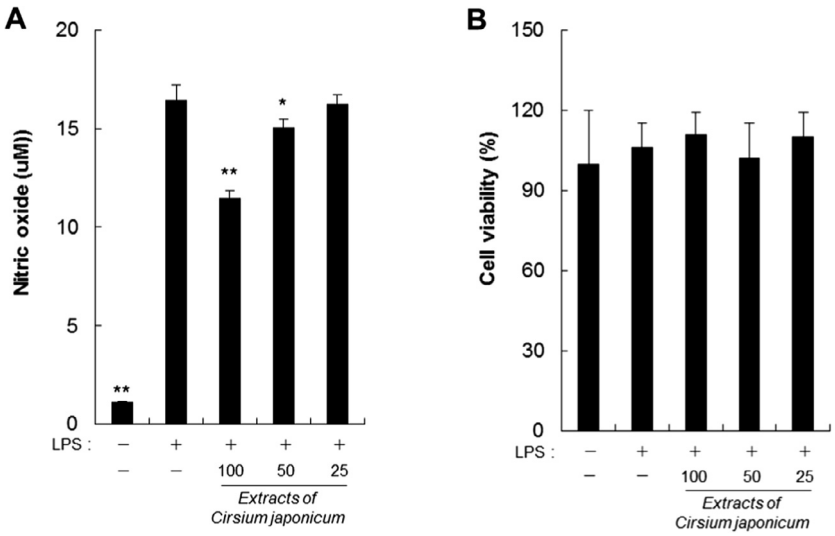

Fig. 1. Effect of ethanol extracts of *Cirsium japonicum* var. *maackii* Maxim. on nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. RAW264.7 cells (4×10^5/well, 24-well plate) were treated with extracts of *C. japonicum* var. *maackii* Maxim. at varying concentrations (25–100 μg/mL) for 1 h, and then stimulated with LPS (50 ng/mL) for 20 h. Cell supernatants were collected and then assayed for nitric oxide with Griess reagent (A). Following treatment, 1/10 diluted EZ-cytox solution was applied to the same cell plate, and further incubated while cytotoxicity effects were evaluated. The percent cell viability was compared to that of the control, which corresponded to cells treated with only medium (B). Results are expressed as the mean ± SD of duplicate experiments. Statistical significance was determined using the two tailed Student's *t*-test, with $^{**}P < 0.01$ or $^{*}P < 0.05$ accepted as significant.

var. *maackii* ethanol extract (ICF-1) and cirsimaritin are still unclear. Therefore, we investigated the inhibitory effects of ICF-1 and its flavonoid, cirsimaritin, on inflammation and further analyzed the molecular mechanisms responsible.

As shown in Fig. 1A, ICF-1 inhibited LPS-induced NO production in a concentration-dependent manner (50–100 μg/mL), with 25 μg/mL showing no inhibitory effect on NO production in RAW264.7 cells. No cell cytotoxicity was observed with the same concentration range of ICF-1 (Fig. 1B).

Lipopolysaccharide (LPS), also known as endotoxin, plays a role as an inflammation inducer in macrophages. In response to LPS, macrophages produce various pro-inflammatory mediators, such as NO, TNF-α, IL-1β, IL-6, and prostaglandin E2 (PGE2). Uncontrolled secretion of these mediators induces inflammation. Therefore, inhibition of a pro-inflammatory mediator is important to curtail an inflammatory disorder.[16]

The cirsimaritin (Fig. 2A) content analyzed by HPLC was 6.240 ± 0.070 mg/g ICF-1. Next, we investigated whether cirsimaritin, a major flavonoid of ICF-1, displays anti-inflammatory activity in RAW264.7 cells. Therefore, we first evaluated IC50 of cirsimaritin, which is calculated 110.97 μg/mL in RAW264.7 cells (Fig. 2B). Next, we treated cirsimaritin for 1 h then treated LPS for another 20 h, analyzed its cytotoxicity in RAW264.6 cells. Because LPS possess cell proliferation activity, it is necessary to confirm cytotoxicity during anti-inflammatory assay in RAW264.7 cells. As shown in Fig. 2C, treatment of cirsimaritin and LPS showed no cytotoxicity at a concentration of 1–12 μg/mL in RAW264.7 cells. In addition, 1–12 μg/mL of cirsimaritin enhanced cell proliferation in RAW264.7 cells. iNOS is an enzyme for NO production, and LPS strongly induced iNOS expression in RAW264.7 cells. However, cirsimaritin treatment completely abolished LPS-induced iNOS expression at concentrations of 5–10 μg/mL (Fig. 2D). This result suggests that cirsimaritin possesses anti-inflammatory activity in RAW264.7 cells. Statistically significant efficacy concentrations were used for additional mechanism studies.

Since pro-inflammatory cytokines, including IL-6 and TNF-α, are crucial markers of inflammation with LPS treatment, we investigated whether cirsimaritin affects LPS-stimulated cytokine production. As shown in Fig. 3A, LPS markedly increased IL-6 and TNF-α production, but pretreatment with cirsimaritin significantly inhibited IL-6 and TNF-α production in a concentration-dependent manner (Fig. 3A). LPS-induced IL-6 production was inhibited by pretreatment with cirsimaritin at a various concentrations (2–10 μg/mL), and IL-6 production was completely blocked with 10 μg/mL cirsimaritin. While TNF-α production was inhibited by cirsimaritin treatment at 5–10 μg/mL, the inhibition was not as strong as that observed with IL-6 production in RAW264.7 cells (Fig. 3A). Activated macrophages secrete large amounts of pro-inflammatory mediators, such as NO and PGE2, as well as pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β during the inflammatory response.[17] The effects of cirsimaritin on the extracellular release of NO were investigated in LPS-stimulated RAW264.7 cells. Cells that were treated with LPS for 20 h strongly increased NO production, whereas cirsimaritin pretreatment at 2–10 μg/mL for 1 h prior to LPS stimulation inhibited the extracellular release of NO (Fig. 3A). Since cirsimaritin completely abolished IL-6, TNF-α, and NO production in LPS-stimulated RAW264.7 cells, we further examined their gene expression at the mRNA level. The results in Fig. 3B show that the suppression of gene expression with cirsimaritin treatment paralleled its effects on secreted proteins.

LPS binds Toll-like receptor 4 (TLR4), a membrane receptor of macrophages, and activates numerous intracellular signaling events, such as NF-κB-inducing kinase (NIK), IKK, and MAPKs.[18] These proteins regulate transcription factors, including AP-1 and NF-κB, to control target gene expression. In resting cells, the transcription factor, NF-κB, frequently composed of p50 and p65 subunits, exists in the cytosol, bound to IκB. When cells are stimulated, activated IKKβ phosphorylates IκBα on Ser32/36, leading to degradation of the latter in the proteasome. The degradation of IκBα allows nuclear localization of NF-κB, which activates transcription of target genes.[19] When cells were treated with LPS, phosphorylation of IκBα was increased, and IκBα was degraded for 1 h. However, pretreatment with cirsimaritin inhibited LPS-induced

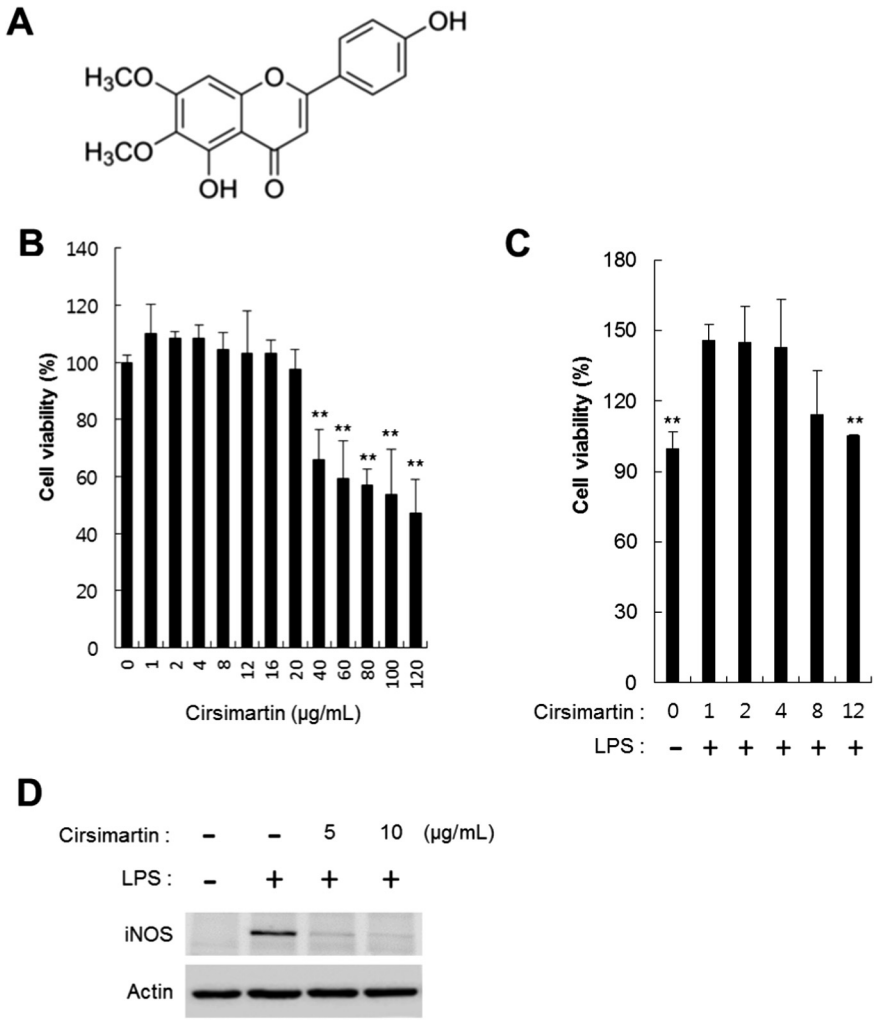

Fig. 2. Effect of cirsimaritin on iNOS expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Chemical structure of cirsimaritin (A). RAW264.7 cells (1×10^5/well, 96-well plate) were treated with cirsimaritin (0–120 μg/mL) for 20 h, and then 10 μL of EZ-cytox reagent added, and cells were further incubated for 4 h. Cell viability was measured by microplate reader (Filtermax F5, Molecular Devices, USA). The percent cell viability was compared with that of the negative control, which corresponded to cells treated with only medium (B). RAW264.7 cells (4×10^5/well, 24-well plate) were treated with cirsimaritin at varying concentrations (1–12 μg/mL) for 1 h, and then stimulated with LPS (100 ng/mL) for 20 h. Then, 50 μL of EZ-cytox reagent added and incubated for another 2 h. The cells viability was measured by microplate reader (C). RAW 264.7 macrophages (2.0×10^6 cells/6-cm dish) were treated indicated concentration of cirsimaritin (5–10 μg/mL) for 1 h, then stimulated LPS (100 ng/mL) for 20 h (D). Whole-cell lysates were then immunoblotted with the iNOS antibody on the left side of each panel. The level of β-actin was measured as an internal loading control. Results are expressed as the mean ± SD of duplicate experiments. Statistical significance was determined using the two tailed Student's *t*-test, with $^{**}P < 0.01$ accepted as significant.

IκBα phosphorylation and degradation (Fig. 4A). Furthermore, since the Akt pathway is involved in LPS activation of signaling pathways and expression of inflammatory mediators in RAW 264.7 cells, we examined the effect of cirsimaritin on phosphorylation of Akt. LPS-induced phosphorylation of Akt was completely suppressed by cirsimaritin treatment (Fig. 4A). However, cirsimaritin treatment did not inhibit MAPKs pathway such as JNK, ERK and p38 (Fig. 4A). Next, we analyzed transcription factors, such as c-fos and signal transducer and activator of transcription 3 (STAT3). c-fos is an AP-1 family member that is downstream of MAPKs. STAT3 is an important molecule for IL-6 receptor signaling and IL-6 autocrine signaling in cells. Cirsimaritin treatment strongly decreased phosphorylation of c-fos and STAT3 in a concentration-dependent manner (Fig. 4B). As we described Fig. 4A, the phosphorylation of MAPKs did not affected by cirsimaritin, inhibition of the phosphorylation of c-fos and STAT3 seem to be a secondary effect originated from strong inhibition of cytokine production by cirsimaritin in RAW264.7 cells (Fig. 4B).

In summary, the pathogenesis of inflammation is a complex processes, involving cytokines and pro-inflammatory genes. Macrophages are an important part of the innate immune response, the first line of host defense against pathogens before adaptive immune responses begin. LPS promotes production of IL-1, IL-6, TNF-α, iNOS, and cyclooxygenase-2 (COX-2) in macrophages. Reduction of inflammatory mediators is an effective method to relieve macrophage-induced inflammation and its symptoms. In this study, we showed that cirsimaritin inhibited LPS-induced NO production, and its flavonoid, cirsimaritin, inhibited iNOS expression in RAW264.7 cells. Cirsimaritin suppressed LPS-induced IL-6, TNF-α, and NO production, and cytokine mRNA levels were inhibited in a concentration-dependent manner. Moreover, cirsimaritin abolished LPS-induced IκBα phosphorylation, IκBα degradation, and activation of transcriptional factors, such as c-fos and STAT3. Collectively, these results suggest that cirsimaritin possesses anti-inflammatory activity and is a promising therapeutic candidate for anti-inflammatory treatment.

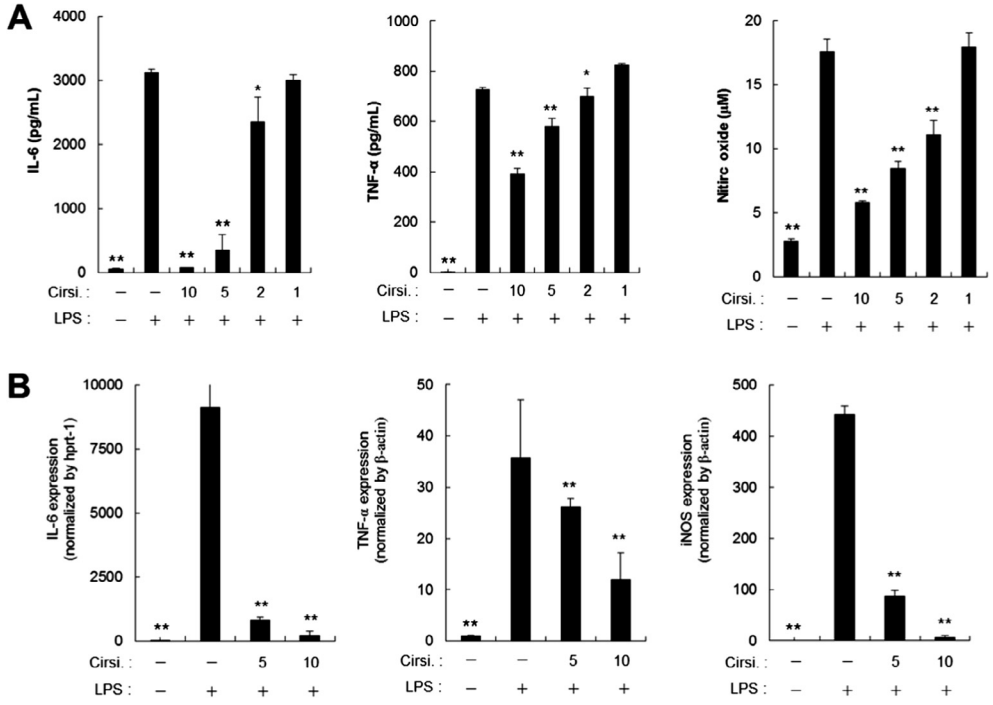

Fig. 3. Effect of cirsimaritin on interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. RAW264.7 cells (4×10^5/well, 24-well plate) were treated with cirsimaritin at varying concentrations (1–10 μg/mL) for 1 h, and then stimulated with LPS (100 ng/mL) for 20 h. Cell supernatants were collected and then analyzed by ELISA kit for cytokines (IL-6 and TNF-α) and with Griess reagent for nitric oxide (A). RAW 264.7 macrophages (2.0×10^6 cells/6-cm dish) were treated with the indicated concentrations of cirsimaritin (5–10 μg/mL) for 1 h, and then stimulated with LPS (100 ng/mL) for 4 h. IL-6, TNF-α, and inducible nitric oxide synthase (iNOS) mRNA expression levels were measured by qRT-PCR. Results are expressed as the mean ± SD of triplicate experiments. Statistical significance was determined using the two tailed Student's *t*-test, with $^{**}P < 0.01$ or $^{*}P < 0.05$ accepted as significant.

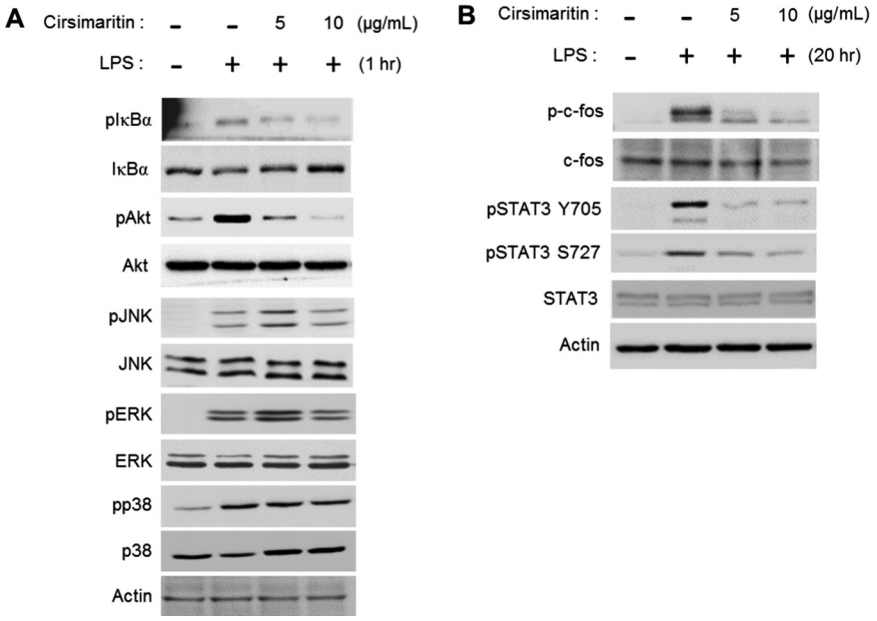

Fig. 4. Effect of cirsimaritin on phosphorylation of IκB, Akt in RAW264.7 cells. RAW 264.7 macrophages (3.0×10^6 cells/6-cm dish) were treated with the indicated concentrations of cirsimaritin (5–10 μg/mL) for 1 h, and then stimulated with LPS (100 ng/mL) for 1 h (A) or 20 h (B). Whole-cell lysates were immunoblotted with the specific antibodies indicated on the left side of each panel. The level of β-actin was measured as an internal loading control. Results are representative blots of duplicate experiments.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.05.051.

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