

Full Paper

Honokiol Inhibits the Progression of Collagen-Induced Arthritis by Reducing Levels of Pro-inflammatory Cytokines and Matrix Metalloproteinases and Blocking Oxidative Tissue Damage

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Abstract. Plant-derived compounds with potent anti-inflammatory activity have attracted a great deal of attention as a source for novel anti-arthritic agents with minimal side effects. We attempted to determine the anti-arthritic effects of orally administered honokiol isolated from *Magnolia* species. The oral administration of honokiol inhibited the progression and severity of type II collagen (CII)-induced arthritis (CIA) by reducing clinical arthritis scores and paw swelling. The histological analysis demonstrated preserved joint space; and the immunohistochemical data showed that the levels of interleukin (IL)-17, matrix metalloproteinase (MMP)-3, MMP-9, MMP-13, and receptor activator for nuclear factor- κ B ligand, as well as nitrotyrosine formation, were substantially suppressed in the honokiol-treated CIA mice. The elevated serum levels of tumor necrosis factor- α and IL-1 β in the CIA mice were also restored to control levels via honokiol treatment. In the CIA mice, honokiol inhibited CII- or lipopolysaccharide-stimulated cytokine secretion in spleen cells, as well as CII-stimulated spleen cell proliferation. Furthermore, honokiol treatment reduced CIA-induced oxidative damage in the liver and kidney tissues of CIA mice. Collectively, the oral administration of honokiol inhibited CIA development by reducing the production of pro-inflammatory cytokines, MMP expressions, and oxidative stress. Thus, honokiol is an attractive candidate for an anti-arthritic agent.

Keywords: type II collagen-induced arthritis, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), matrix metalloproteinase (MMP), oxidative damage

Introduction

Rheumatoid arthritis (RA) affects approximately 1% of the world's population, at a female/male ratio of 3/1. This disease can occur at any age, but is most common among those aged 40 – 70 years, and its incidence increases with age (1, 2). RA is generally considered to be a human autoimmune disease characterized by chronic inflammation in multiple joints and causes the destruction of periarticular bone, resulting in bone erosion and

functional disabilities (3). In RA, synovial tissues represent hyperplasia via infiltrations of lymphocytic and synovial lining cells, as well as vascular proliferation (4). The cytokines generated by these infiltrating cells in synovial tissues have been directly implicated in the immune-regulatory and tissue-destructive events that underlie the clinical presentation and progression of RA by promoting autoimmunity, maintaining chronic inflammatory synovitis, and driving the destruction of adjacent joint tissues. Cytokines are, therefore, currently being tested as targets in the treatment of RA (5).

The destruction of articular cartilage in RA is closely associated with the increased activity of cytokine-induced matrix metalloproteinase (MMPs), a family of matrix-

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degrading enzymes capable of degrading all major extracellular matrix (ECM) components, and which are important mediating factors in inflammatory and connective tissue diseases (6). Interleukin (IL)-1 and tumor necrosis factor (TNF) stimulate the induction of MMP-1, MMP-3, MMP-9, and MMP-13 in synovial fibroblasts, macrophages, and chondrocytes; and IL-17 induces MMP-1, MMP-3, and MMP-13 expression in synovial fibroblasts (7). Among these MMPs, the interstitial collagenases, MMP-1 and MMP-13, induce the degradation of collagen type II (CII), an early irreversible event that occurs within the superficial cartilage layer (8). MMP-3 digests proteoglycans and collagen types IX and X and is thus considered a key enzyme in the pathological destruction of cartilage (9). Additionally, the gelatinase subfamily, including MMP-2 and MMP-9, is also of considerable import in arthritis (10). MMP-9 degrades the fragmented interstitial collagen and collagen types IV and V.

In many joint diseases, oxygen-derived free radicals are generated in large quantities at the inflammation site, together with pro-inflammatory factors, and thus are considered mediators of tissue damage (11, 12). Studies conducted with the synovial fluids and tissues of RA patients have reported increased oxidative enzyme activity, along with reduced anti-oxidant levels, and oxidative damage in cartilage, extracellular collagen, and DNA (13). TNF- α overproduction is believed to induce increased reactive oxygen species (ROS) release in RA patients; this supposition is supported by recent studies demonstrating that the anti-TNF- α antibody infliximab or the TNF- α inhibitor Etanercept reduces the levels of oxidative stress markers in RA patients (14, 15). Additionally, higher levels of nitric oxide (NO) are expressed in RA cartilage as compared with normal cartilage, and this results in a diminished synthesis of ECM components, MMP activation, inhibitions of proliferation, and the induction of apoptotic death in chondrocytes (16, 17). Thus, blockade of ROS and NO production, as well as pro-inflammatory cytokines, might also prove important in controlling disease severity by ameliorating joint inflammation and connective tissue damage in RA patients.

Honokiol is a small-molecule polyphenolic compound that is isolated from the stem bark of *Magnolia obovata* THUNB and *Magnolia officinalis* REHD (Magnoliaceae) that has been used without notable side effects in traditional Oriental medicine for the treatment of anxiety, neurosis, and asthma (18, 19). Honokiol evidences a variety of pharmacological activities, including antioxidative (20–22), anti-inflammatory (23, 24), and antitumor activities (25, 26). In this study, we attempted to determine whether the oral administration of honokiol might prevent the progression of collagen-induced arthritis

(CIA), which has been used as an animal model for studies of RA pathogenesis and to evaluate potential therapeutic agents (27). Furthermore, we found that the administered honokiol inhibited joint and tissue damages by reducing the levels of pro-inflammatory cytokines, MMPs expression, and ROS generation in CIA mice.

Materials and Methods

Materials

Honokiol of 99.0% purity was purchased from Wako Pure Chemical (Tokyo, Lot No. WKL1096) and dissolved in dimethyl sulfoxide (DMSO). Bovine CII, complete Freund's adjuvant (CFA), and incomplete Freund's adjuvant (IFA) were acquired from Chondrex (Redmond, WA, USA). Primary antibodies against MMP-3, MMP-9, receptor activator of nuclear factor- κ B ligand (RANKL), IL-17, and normal mouse immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies of MMP-13 and nitrotyrosine were acquired from Millipore Corporation (Billerica, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA). Polink-1 HRP Goat for the DAB Detection kit, consisting of polymeric HRP-linked anti-goat antibody, chromogen, and substrate, was obtained from Golden Bridge International (Mukilteo, WA, USA). Harris' hematoxylin was purchased from Merck (Darmstadt, Germany). RPMI 1640, fetal bovine serum (FBS), antibiotic-antimycotic mixture containing penicillin and streptomycin, and phosphate-buffered saline (PBS) were all purchased from Gibco BRL (Gaithersburg, MD, USA). Bovine serum albumin (BSA), eosin Y disodium salt, Mayer's hematoxylin, lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, concanavalin A, 2-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), red blood cell-lysing buffer, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Seven- to 9-week-old male DBA/1J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed in a controlled environment ($22 \pm 2^\circ\text{C}$, 12-h light/dark cycle) and provided with standard rodent chow (Central Lab Animal, Seoul, Korea) and tap water. All of the animal experiments conducted herein were conducted in compliance with the guidelines and regulations for the use and care of animals established by Yonsei University (Seoul, Korea).

Induction of CIA and honokiol treatment

Bovine CII at 2 mg/ml was emulsified with an equal

volume of CFA supplemented with *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg/ml. The mice were injected intradermally at the tail base with 100 μ l of the emulsion containing 100 μ g of CII. After 21 days of primary immunization, the mice received boosters with 100 μ g of CII in IFA. To evaluate the effects of honokiol on CIA, the CIA mice were divided into three groups, each containing 5 mice; and vehicle (PBS containing 2% ethanol and 1% Tween 20) or honokiol at 10 or 20 mg/kg body weight (BW) was administered by oral gavage once per day from days 25 to 45. The control mice were not treated with CII or honokiol. CIA development was evaluated via macroscopic scoring and measurements of paw swelling. Arthritis severity was recorded and assessed with a clinical scoring system on a scale of 0–4 for each paw: 0 = no signs of arthritis, 1 = swelling and/or redness in one joint, 2 = swelling and/or redness in more than one joint, 3 = swelling and/or redness in the entire paw, 4 = severe swelling of the entire paw with deformity and/or ankylosis. The total arthritis score of each mouse yielded a maximum score of 16, as the sum of each score of the four limbs. Paw swelling was assessed by measuring the thickness of two hind paws using a digital caliper. On day 46, all mice were sacrificed by anesthesia after blood collection. Knee joints for histological examination, spleens for cytokine level measurements, and livers and kidneys for oxidative stress tests were extracted from the control and CIA mice.

Histological and immunohistochemical examination

The knee joints were removed from all mice at 46 days after primary immunization and then fixed in 10% buffered formalin solution, decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA), and paraffin-embedded. Standard sagittal sections measuring 5 μ m were prepared and stained with hematoxylin and eosin. Histological evaluations were performed independently and blindly using the following scoring system: 0, no inflammation; 1, hyperplasia of the synovial tissue; 2, infiltration of the inflammatory cells; 3, pannus formation and cartilage erosion; and 4, extensive erosion of cartilage and bone.

For immunohistochemical analysis, 5- μ m sections of formalin-fixed knee joint tissues were deparaffinized, rehydrated, and submitted to trypsin-induced antigen retrieval for 20–30 min at 37°C in a humidified chamber. Endogenous peroxidase activity was quenched via the immersion of the slides in 3% H₂O₂ in methanol for 10 min at room temperature. The specimens were handled for 30 min with 3% BSA in PBS and then incubated with primary antibody against MMP-3, MMP-9, MMP-13, IL-17, RANKL, or nitrotyrosine. After three washings

with PBST (PBS containing 0.05% Tween-20), the specimens were incubated for 20 min with HRP-conjugated antibodies at room temperature and stained with DAB dye. The sections were then counterstained with Mayer's hematoxylin.

Measurement of spleen cell proliferation

Spleens were isolated from all mice at 46 days and dissociated into single cells using cell strainers. After treatment with red blood cell-lysing buffer and two washings in ice-cold PBS, single cells were resuspended in RPMI 1640 medium containing 50 μ M 2-mercaptoethanol and 10% FBS. Single spleen-cell suspensions (2×10^5 cells/well) were incubated in 96-well plates for 72 h at 37°C in 5% CO₂ and then stimulated with 50 μ g/ml of denatured CII or 1 μ g/ml of concanavalin A. Cell proliferation was evaluated with a BrdU cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany).

Measurement of TNF- α and IL-1 β levels

To obtain sera, blood samples taken via intracardiac puncture from the control and CIA mice were permitted to clot for 2 h at room temperature and then centrifuged for 20 min at $2,000 \times g$ at 4°C. On the other hand, suspensions of spleen cells (2×10^6 cells/well) isolated from mice were incubated in RPMI medium alone or in RPMI medium with 50 μ g/ml denatured-CII or 5 μ g/ml LPS, for 48 h at 37°C. The levels of TNF- α and IL-1 β in each serum and the conditioned spleen cell media were measured using specific ELISA kits in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Preparation of tissue homogenates

Liver and kidney tissues isolated from the control and CIA mice were immediately washed with ice-cold saline to remove the blood. The sliced tissues (100 mg) were subsequently homogenized in 1.0 ml of cold 1.15% KCl buffer (pH 7.4) containing 100 mM PMSF. Tissue homogenates were centrifuged for 10 min at $800 \times g$ at 4°C, and the supernatants were collected in order to measure the levels of lipid peroxidation, reduced glutathione (GSH), and antioxidant enzymes. Total protein concentrations in the supernatants were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Measurement of lipid peroxidation and GSH levels

The levels of malondialdehyde (MDA), a good indicator of lipid peroxidation, and GSH in tissue homogenates were measured as described previously (28). MDA content was detected in the form of thiobarbituric acid reactive substances (TBARS) at 532 nm. 1,1,3,3-Tetrame-

thoxypropane was utilized to establish a standard curve, and the final MDA concentration is expressed as nmol MDA per mg tissue protein. GSH levels were estimated at 412 nm colorimetrically after complex formation with DTNB and expressed as nmol per mg tissue protein.

Measurement of antioxidant enzyme activities

Superoxide dismutase (SOD) activity was measured using a commercially available assay kit in accordance with the manufacturer's protocols (Fluka, Buchs, Switzerland). The determination of catalase activity was predicated on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde generated was measured with purpald as a chromogen at 540 nm (28). Enzyme activity was expressed as units per mg tissue protein. One unit (U) was defined as the amount of enzyme required to induce the formation of 1 nmol of product per min at 25°C.

Statistical analyses

Data were expressed as the means \pm standard error (S.E.M.) and analyzed via one-way ANOVA with multiple comparisons followed by Tukey's test. The data in Fig. 1 were estimated via two-way ANOVA. *P*-values of less than 0.05 were considered statistically significant.

Results

Effect of honokiol on CIA progression in mice

In order to evaluate the effect of honokiol on CIA, DBA/1J mice were immunized with bovine CII in CFA, then boosted with CII in IFA on day 21 after primary immunization. Oral administration of honokiol was initiated on day 25, when the first clinical signs of disease, such as redness or swelling, occurred, and dosage was continued once daily for 21 days. CII immunization induced CIA and gradually exacerbated arthritic symptoms. On day 45, all of the vehicle-treated mice evidenced CIA with a mean arthritis score of 7.5 ± 1.3 and a mean paw thickness of 2.73 ± 0.03 mm. Treatment with 10 and 20 mg/kg of honokiol dose-dependently inhibited the swelling of the paws containing the footpad, ankle and wrist joints, and digits, thereby significantly reducing arthritis scores to 3.8 ± 1.0 and 2.6 ± 0.8 (Fig. 1A), respectively, and paw thickness to 2.65 ± 0.03 and 2.57 ± 0.02 mm, respectively (Fig. 1B).

Effect of honokiol on the histopathological changes and the levels of MMPs, RANKL, IL-17, and nitrotyrosine in the joints of CIA mice

H&E staining to verify the clinical assessments revealed that the knee joint tissues of the vehicle-treated

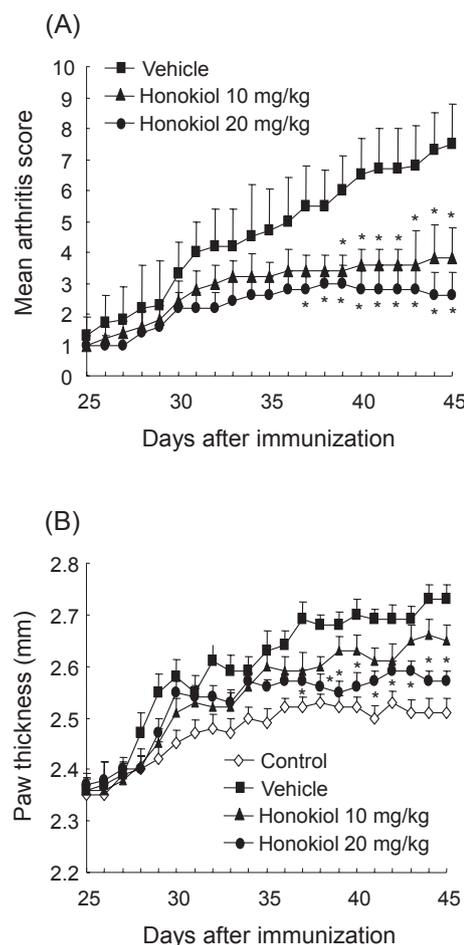


Fig. 1. Effect of honokiol on the progression and severity of CIA in mice. Honokiol at 10 and 20 mg/kg was orally administered to CII-immunized mice once daily for 21 days after the first clinical signs of CIA began. The severity of arthritis was evaluated by clinical arthritis score (A) and hind paw thickness (B). Data are expressed as the mean \pm S.E.M. of 7 mice per group. **P* < 0.05 vs. vehicle-treated CIA mice.

CIA mice exhibited noticeable pathologic changes, including synovial hyperplasia, a large number of infiltrating inflammatory cells, and extensive pannus formation. Completely disrupted joint spaces with indiscriminant cartilage remodeling and bone erosion were also noted in the vehicle-treated CIA mice. However, orally administered honokiol inhibited these histopathological events in a dose-dependent manner, thus inducing a substantial recovery in the joints of CIA mice, as shown in Fig. 2.

To determine the mechanism by which honokiol treatment exerts an inhibitory effect on cartilage and bone destruction, we immunohistochemically analyzed the levels of CIA-related MMPs, IL-17, RANKL, and nitrotyrosine in the knee joints of the control mice and CIA mice with or without honokiol treatment. Substantially enhanced levels of MMP-3, MMP-9, MMP-13, IL-17,

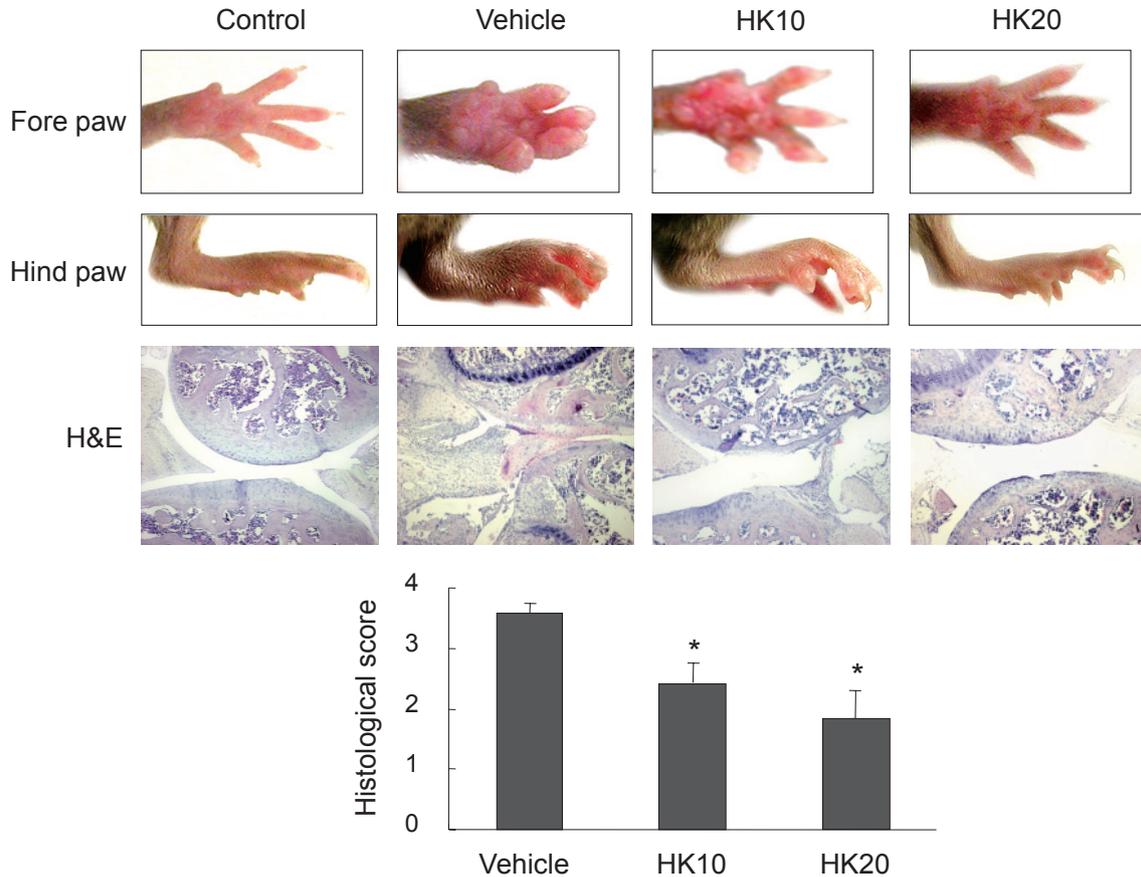


Fig. 2. Histopathologic analysis in the joints of CIA mice with or without honokiol treatment. Following the onset of CIA, honokiol at 10 mg/kg (HK10) and 20 mg/kg (HK20) was orally administered to CII-immunized mice once daily for 21 days. On day 46, fore and hind paws of control mice and CIA mice treated with vehicle or honokiol were observed, and the photographs shown are representative from control mice and CIA mice with or without honokiol treatment (upper and middle). Joint tissues from all mice were stained with H&E (lower). Photographs are representative of at least three independent experiments. Histological scores are expressed as the mean \pm S.E.M. of 7 mice per group in three independent experiments. * $P < 0.05$ vs. vehicle-treated CIA mice.

and RANKL were observed in the vehicle-treated CIA mice, but were clearly inhibited in the honokiol-treated CIA mice. Additionally, honokiol treatment inhibited CIA-induced nitrotyrosine formation (Fig. 3).

Effect of honokiol on serum levels of TNF- α and IL-1 β in CIA mice

To elucidate the mechanisms underlying the reduction in the severity of CIA after honokiol treatment, we also assessed the levels of pro-inflammatory cytokines in the blood sera of the control and all of the CIA mice. The significantly increased levels of TNF- α (Fig. 4A) and IL-1 β (Fig. 4B) in the vehicle-treated CIA mice were dose-dependently reduced by the honokiol treatment and recovered almost to control levels at a honokiol dosage of 20 mg/kg.

Effect of honokiol on the pro-inflammatory immune responses in CIA mice

We further evaluated cell proliferation or/and cytokine secretions when the spleen cells of control and all CIA mice were stimulated with concanavalin A, denatured CII, or LPS. Denatured CII stimulation resulted in profound cell proliferation in the spleen cells of vehicle-treated CIA mice, but did not induce significant cell proliferation in the spleen cells of the honokiol-treated CIA mice. Stimulation with concanavalin A did not affect the proliferation of spleen cells from the CIA mice (Fig. 5A). The TNF- α (Fig. 5B) and IL-1 β levels (Fig. 5C) in the conditioned media of spleen cells from vehicle-treated CIA mice were elevated considerably by stimulation with denatured CII or LPS, but these CII- and LPS-induced elevations in cytokine levels were suppressed in the honokiol-treated CIA mice.

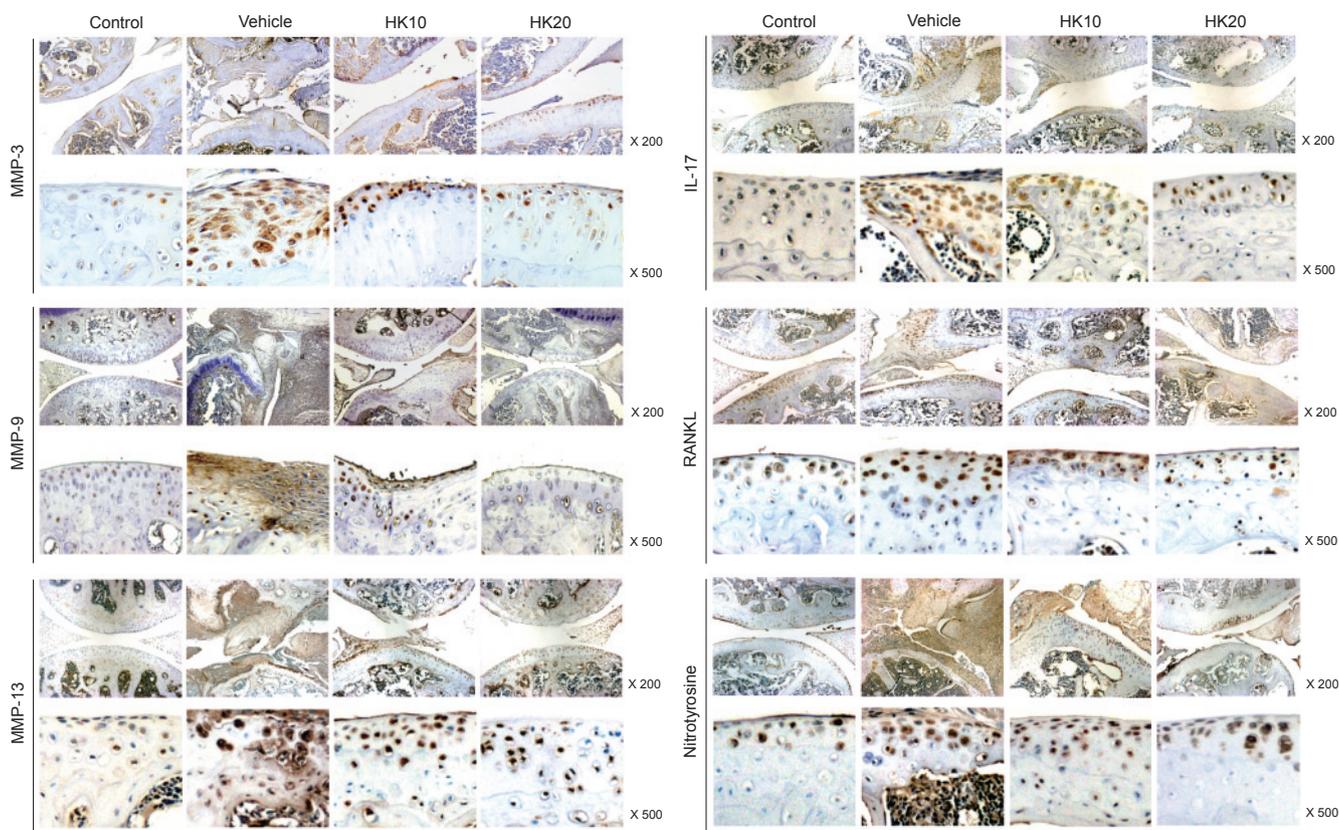


Fig. 3. Immunohistochemical analysis in the joints of CIA mice with or without honokiol treatment. On day 46, joint tissues from control mice and CIA mice treated with vehicle or honokiol were immunohistochemically stained for the detection of MMP-3, MMP-9, MMP-13, IL-17, RANKL, and nitrotyrosine. Representative data from control mice and CIA mice treated with vehicle or honokiol are shown.

Effect of honokiol on oxidative damages in liver and kidney tissues of CIA mice

To evaluate the effect of honokiol on CIA-induced oxidative tissue damages, the levels of MDA and GSH, as well as the activity of antioxidant enzymes, were measured in the homogenates of liver and kidney tissues from the control and all of the CIA mice. While the levels of MDA were increased in the tissues from the vehicle-treated CIA mice (Fig. 6A), the levels of GSH were reduced (Fig. 6B) as compared with the control mice. Additionally, catalase (Fig. 6C) and SOD activity (Fig. 6D) were also reduced in the vehicle-treated CIA mice. Honokiol at dosages of 10 and 20 mg/kg lowered the levels of CIA-induced MDA and recovered the GSH levels and antioxidant enzyme activity in the CIA mice to the control level.

Discussion

RA is a complicated refractory autoimmune disease characterized by chronic inflammatory and destructive events such as joint pain and swelling, synovial hyper-

plasia by inflammatory cells infiltrated in the synovium and synovial fluid, pannus formation, and ultimately, joint malformation via the destruction of articular cartilage and erosion of subchondral bone (29). Time-tested standard treatments, including immunosuppressive disease-modifying anti-rheumatic drugs and non-steroidal anti-inflammatory drugs, exert well-known but significant toxic side effects. Therefore, plant-derived compounds with potent anti-inflammatory activity have become the focus of increased attention as a possible source for novel anti-arthritis agents with minimal side effects. The results of recent studies have shown that honokiol inhibits NO synthesis and TNF- α expression in lipopolysaccharide-activated macrophages (30) and RANKL-induced osteoclastogenesis in RAW264.7 cells (31). Intra-peritoneal injection of honokiol at a dosage of 3 mg/mouse per day ameliorated murine CIA by suppressing the production of anti-CII-specific antibodies and CD40-mediated signaling in activated B cells (32). In the present study, in order to further characterize the anti-arthritis effects and mechanisms underlying the activity of orally administered honokiol, we focused on CIA-induced pro-

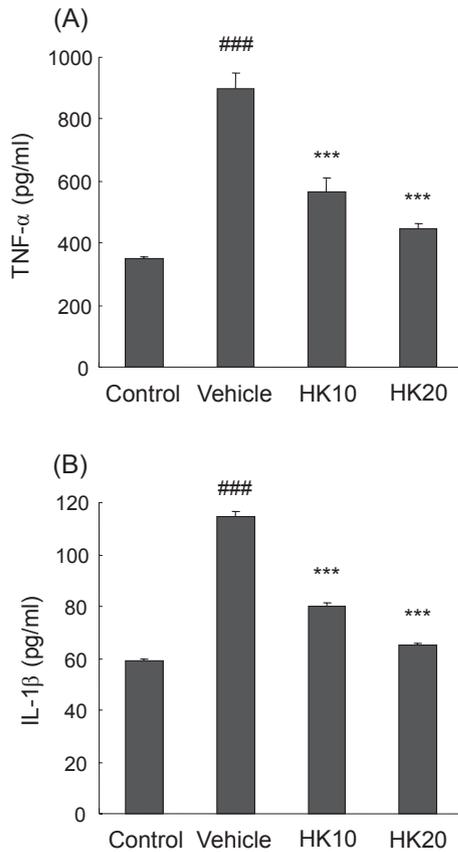


Fig. 4. Effect of honokiol on the serum TNF- α and IL-1 β levels in CIA mice. On day 46, the levels of TNF- α (A) and IL-1 β (B) were measured via ELISA assay in the serum of each CIA mice with or without honokiol at 10 mg/kg (HK10) and 20 mg/kg (HK20). Data are expressed as the mean \pm S.E.M. of 7 mice per group. ### P < 0.001 vs. control mice, *** P < 0.001 vs. vehicle-treated CIA mice.

inflammatory cytokines, MMP expression, and oxidative damages via the immune responses of T cells and macrophages.

CIA is a valid animal model with many of the clinical and pathological features of human RA. In DBA/1J mice immunized with CII/CFA, stimulated dendritic cells (DCs) take up the CII protein and migrate to draining lymph nodes, presenting the processed CII peptide on the appropriate MHC class II molecules to naive T cells. The engagement of the activated DCs and T cells is accompanied by the activation of naive B cells. Consequently, the binding and accumulation of the resultant anti-CII antibodies initiate inflammatory responses in the articular region via the activation of the complement cascade, recruiting a variety of immune cells (33). Among the recruited synovial immune cells, IL-17-secreting T cells have recently become the focus of renewed attention as a key contributor to the pathogenesis of arthritis. IL-17 strongly stimulates TNF- α and IL-1 β production by macrophages, IL-6 and PGE₂ by synovial fibroblasts, and

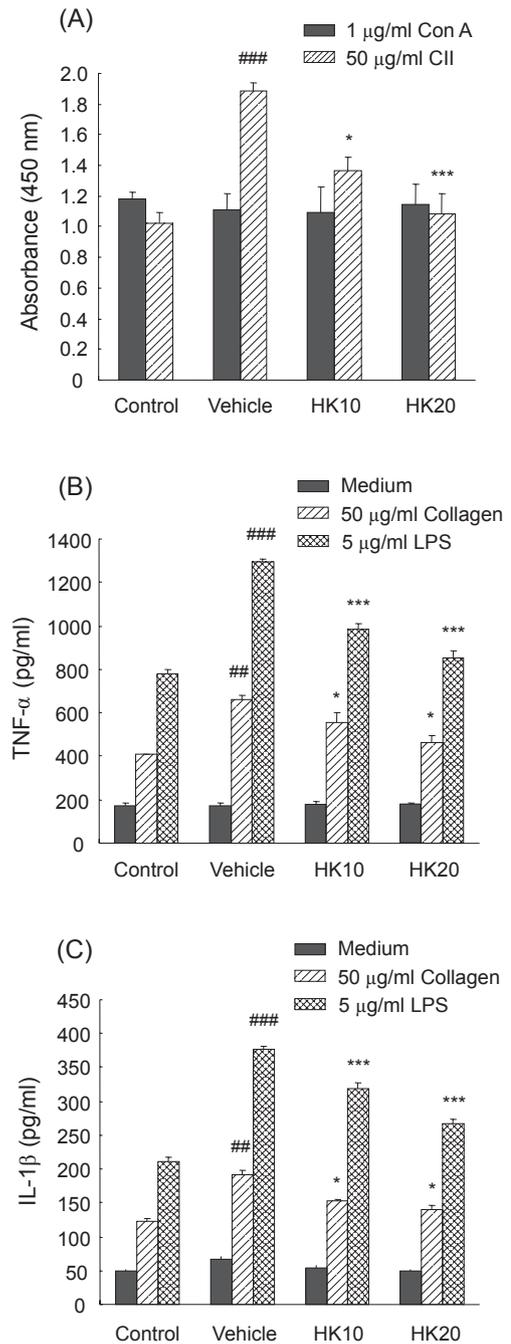


Fig. 5. Effect of honokiol on CII- or LPS-stimulated immune responses of spleen cells in CIA mice. On day 46, spleens were isolated from control mice and all CIA mice. Single-cell suspensions from spleens were acquired from the control mice and all CIA mice. The proliferation of spleen cells stimulated with 1 μ g/ml of concanavalin A or 50 μ g/ml of denatured CII for 72 h was evaluated with a BrdU cell proliferation ELISA kit (A), and the TNF- α (B) and IL-1 β (C) levels were measured in the conditioned media of spleen cells stimulated with 50 μ g/ml of denatured CII or 5 μ g/ml of LPS for 48 h, using each specific ELISA kit. Data are expressed as the mean \pm S.E.M. of 7 mice per group. ### P < 0.01, #### P < 0.001 vs. control mice; * P < 0.05, *** P < 0.001 vs. vehicle-treated CIA mice.

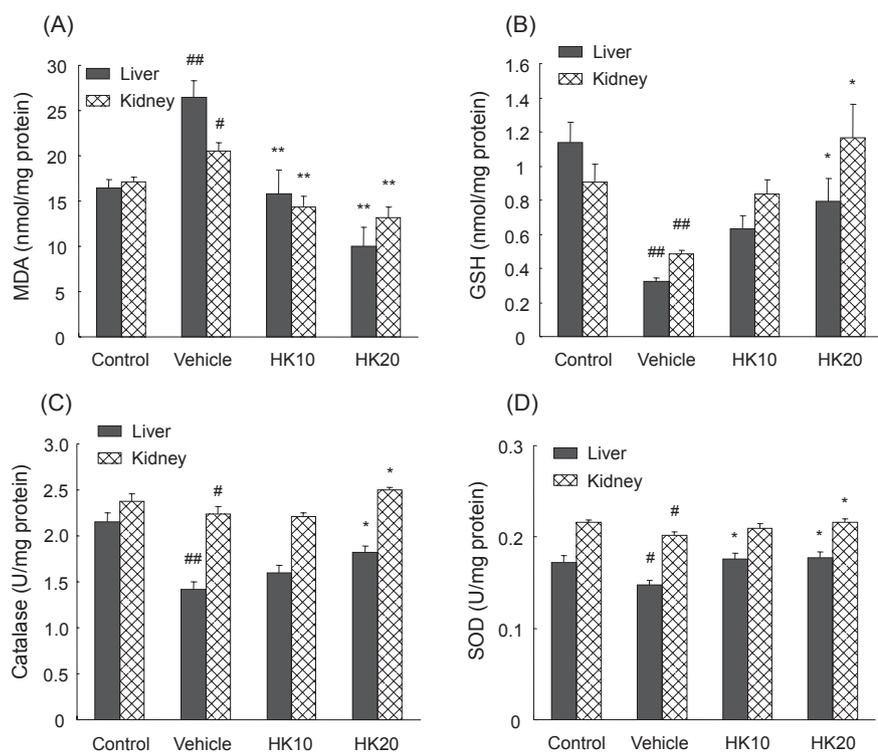


Fig. 6. Effect of honokiol on oxidative tissue damages in CIA mice. Liver and kidney tissues were collected from all mice on day 45, homogenized in 1.15% KCl buffer, and centrifuged. MDA (A) and GSH (B) levels as well as activities of antioxidant enzymes catalase (C) and SOD (D) in the homogenates were measured as described in the Materials and Methods section. Data is expressed as the mean \pm S.E.M. of 7 mice per group. [#] $P < 0.05$, ^{##} $P < 0.01$ vs. control mice; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. vehicle-treated mice.

NO in the articular chondrocytes (34, 35). IL-17 induces MMP-2, MMP-3, MMP-9, and MMP-13 expression and exerts synergistic effects with IL-1 and TNF- α in inducing the expression of other pro-inflammatory cytokines and joint damage mediated by MMPs (36, 37). The increased MMP levels in RA have been closely associated with cartilage destruction via the degradation of the major ECM components, CII and aggrecan (7, 38, 39). In addition to the MMPs, IL-17 induces NO production-degenerating chondrocytes (40), and RANKL expression, stimulating osteoclastogenesis and subsequent bone erosion (41–43).

In this study, the oral administration of honokiol was shown to ameliorate the progression of CIA via a reduction in clinical arthritis scores and paw swelling. Body weights in mice were reduced by CIA induction, but were recovered by honokiol treatment. These changes in body weight were not statistically significant. CIA did not affect behaviors and signs of mice (data not shown).

The results of histological analysis confirmed its anti-arthritis activity by showing the preserved joint space resulting from the substantial reduction of chronic inflammation in synovial tissues, pannus formation, and cartilage destruction in the joints of honokiol-treated CIA mice as compared with what was observed in vehicle-treated CIA mice. Additionally, immunohistochemical data demonstrated that the levels of CIA-induced proteins, including IL-17, MMP-3, MMP-9, MMP-13, and

RANKL, and the formation of nitrotyrosine as a marker of NO production, were suppressed substantially in the honokiol-treated CIA mice. The abnormally elevated serum levels of TNF- α and IL-1 β in vehicle-treated CIA mice were found to be restored to control levels in the honokiol-treated mice. TNF- α and IL-1 β are essential to cartilage degradation via the inhibition of matrix synthesis, as well as via the induced expression of matrix-degrading enzymes, most notably MMPs (44). Our results show that the oral administration of honokiol to CIA mice inhibits inflammatory responses, cartilage destruction, and bone erosion by blocking IL-17, MMPs, RANKL, and NO production in the inflamed joints and by reducing serum levels of TNF- α and IL-1 β .

To determine whether honokiol treatment *in vivo* affected cell-mediated immunity to collagen, cell proliferation and the levels of pro-inflammatory cytokines were measured in CII- or LPS-stimulated spleen cells isolated from control and CIA mice in the presence or absence of honokiol treatment. The spleen is the principal site of adaptive immune responses to blood-borne antigens, owing to an abundance of resident phagocytes and lymphocytes. Spleen cells are stimulated with CII and concanavalin A to activate T cells or LPS to potentially activate macrophages (45). The spleens from the vehicle-treated CIA mice in our study were substantially enlarged as compared to those from the controls (data not shown), as evidence that CII augmented immune responses in the

CIA mice. Cell proliferation stimulated by CII, not concanavalin A, was elevated in the spleen cells from the vehicle-treated CIA mice, thereby suggesting that the T cell-mediated immune responses in our CIA mice occurred in an antigen-specific manner. Moreover, CII- or LPS-stimulated levels of TNF- α and IL-1 β pro-inflammatory cytokines, which are linked to joint inflammation of RA (46), were enhanced significantly in the spleen cells from vehicle-treated CIA mice. Honokiol treatment inhibited CII- or LPS-stimulated cytokine secretion in the spleen cells of CIA mice, as well as CIA-induced spleen enlargement and CII-stimulated proliferation of spleen cells from CIA mice. These results indicate that honokiol treatment inhibits murine CIA via the modulation of immune responses by activated T cells and macrophages. Additionally, the reduced cytokine levels noted in the CII/LPS-stimulated spleen cells explain the reduction in serum cytokine levels observed in the honokiol-treated mice.

Radical species with oxidative activity, including ROS and NO, represent the mediators and effectors of the loss of articular cartilage, resulting in an irreversible impairment of joint motion (47, 48). Increased oxidative stress in RA patients is associated with reduced antioxidant capacity as well as cytokine-stimulated ROS and NO production (49). Thus, the control of ROS and NO levels is a key target for reducing disease severity, joint remodeling via MMP production, and tissue damage. As mentioned above, the increased NO production in murine CIA and its reduction by honokiol treatment was confirmed via the immunohistochemical detection of nitrotyrosine residues in the joints of CIA mice. Nitrotyrosine, which is formed when tyrosine is oxidized in the presence of toxic peroxynitrite derived from the superoxide anion and NO, can be utilized as a measure of oxidative damage in vivo. Furthermore, our data demonstrated that honokiol treatment reversed the increase in lipid peroxidation and blocked the decreases in the levels of GSH and the antioxidant enzymes SOD and catalase in the liver and kidney tissues of the CIA mice. These results show that orally administered honokiol attenuates oxidative stress and may consequently protect against joint destruction and tissue damage in CIA mice.

Collectively, the oral administration of honokiol inhibited CIA progression by reducing the production of pro-inflammatory cytokines, the expression of MMPs, and oxidative stress. Therefore, honokiol may have potential as an anti-arthritic agent for clinical application.

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