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Journal of Nutritional Biochemistry 18 (2007) 609–614

Journal of Nutritional Biochemistry

Genistein reduces the production of proinflammatory molecules in human chondrocytes

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Received 9 August 2006; received in revised form 2 November 2006; accepted 9 November 2006

Abstract

Previously, we reported that cartilage is an estrogen receptor (ER) positive tissue and that mRNA levels of ERβ increase in postmenopausal women with osteoarthritis. Based on our findings and those of other investigators, we hypothesized that local rather than circulating estrogen levels negatively affect chondrocyte metabolism and that selective ER modulators (SERM) augment cartilage health. To test the latter part of our hypothesis, we explored the role of genistein, a naturally occurring SERM with high affinity to bind ERβ, in inhibiting the lipopolysaccharide (LPS)-stimulated cyclooxygenase (COX)-2 in chondrocytes. Primary cultures of normal human chondrocytes were treated with three levels of genistein (0, 50, and 100 μM). After 1 h, the genistein-treated cells were stimulated by 1 μg/ml LPS for 24 h. Cells were then harvested, and the cytosolic fraction was isolated for assessment of COX-1 and COX-2 protein levels using Western analysis. Nitric oxide (NO), interleukin-I beta (IL-1β), and human cartilage glycoprotein 39 (YKL-40) production was also measured in cell supernatants. NO and IL-1β were measured as markers of inflammation, and YKL-40 was assessed as a marker of cartilage catabolism. Genistein had no significant effect on either YKL-40 or IL-1β levels. Our data indicate that the LPS-stimulated increases in COX-2 protein level and NO in supernatant are reduced by pretreatment of genistein, whereas COX-1 protein level is not affected by genistein. The ability of genistein to suppress COX-2 but not COX-1 is advantageous because suppressing COX-2 can lead to suppression of proinflammatory molecules. Although genistein suppresses COX-2 production, it does not affect the production of COX-1 enzyme, which is responsible for releasing prostaglandins involved in cellular house-keeping functions such as the maintenance of gastrointestinal integrity and vascular homeostasis.

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Keywords: Isoflavones; Inflammation; SERM; Lipopolysaccharide

1. Introduction

Osteoarthritis (OA) is the most common form of joint disorder associated with aging. It is characterized by changes in subchondral bone and progressive erosion of articular cartilage, resulting in the loss of joint function [1]. Epidemiological and clinical studies [2–5] have linked estrogen deficiency in postmenopausal women to incidence of knee and hip OA. However, the role of estrogen in the pathogenesis of OA is not clear. Several in vivo [6,7] and in vitro [8,9] studies have demonstrated that cartilage is an estrogen-sensitive tissue. Both isoforms of estrogen receptors (ERs), ERα and ERβ, are present in chondrocytes from humans [10,11] and animals [12,13]. The findings of these studies suggest that cartilage is a target tissue for estrogen. This view is further supported by observations that intraarticular injections of estradiol up-regulate both (ERα and ERβ) in condylar cartilage at early stages of OA in ovariectomized rabbits [14]. Although most animal studies [15,16] have indicated that direct administration of estrogen to knee joints increases frequency and severity of OA, not all clinical findings are in agreement with these observations. Therefore, the role of estrogen in the development of OA needs further investigation.

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doi:10.1016/j.jnutbio.2006.11.006

Although the etiology of OA is unclear, it is believed that the production of the pro-inflammatory cytokines in the joints may play a pivotal role in OA pathogenesis. Management of OA symptoms is currently focused on reducing pain and inflammation through nonsteroidal anti-inflammatory drugs (NSAIDs) or other agents. Among the therapeutic options, alternative and complementary approaches such as herbal and nutritional manipulations are becoming popular. Recent studies [17,18] have suggested that plant flavonoids attenuate inflammation and the immune response through their inhibition of important regulatory enzymes involved in arachidonic acid metabolism. Flavonoids are powerful inhibitors of both lipoxygenase and cyclooxygenase (COX)-2 activities [18,19]. These anti-inflammatory properties of flavonoids provide the rationale for investigating the role of isoflavones in conditions such as OA. The findings of our earlier clinical study have shown that patients with osteoarthritis may benefit from consumption of soy isoflavones [20], a rich source of genistein and daidzein. Genistein and daidzein, the prominent soy isoflavones, are structurally similar to the selective ER modulator (SERM) tamoxifen and the synthetic isoflavone ipriflavone. Tamoxifen [15], raloxifene [21], and ipriflavone [22] have all been shown to have beneficial effect on cartilage metabolism and/or alleviation of OA symptoms.

In the present study, we investigated the effect of genistein on COX-1, COX-2, and nitric oxide (NO) production in response to increasing doses of genistein in lipopolysaccharide (LPS)-treated cultured human chondrocytes. Short-term administration of agents to animals or cell cultures [23–26], such as LPS, the primary cell wall component of Gram-negative bacteria, or interleukin-1 beta (IL-1β) initiates the release of a cascade of inflammatory cytokines involved in the innate immune response [27]. For this reason, we used LPS to up-regulate the production of proinflammatory molecules in chondrocytes. We also measured supernatant concentration of IL-1β and the human cartilage glycoprotein 39 (YKL-40), a marker of tissue remodeling produced by chondrocytes [28].

2. Materials and methods

2.1. Cell culture

Normal human chondrocytes (from hip) were purchased from PromoCell (Heidelberg, Germany). Twenty thousand chondrocytes per well were cultured in six-well plates and grown to 80% confluency in complete chondrocyte growth medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 humidified incubator. Genistein (Sigma, St Louis, MO, USA) was dissolved in dimethylsulfoxide and added directly to the culture media in doses of 0, 50, and 100 μM of genistein when cells were 80% confluent. After 1 h of incubation at 37°C in 5% CO_2, 1 μg/ml LPS (Sigma) was added to all groups. Twenty-four hours later, cell culture media and cells were separately collected for use in subsequent analysis. Cells treated only with dimethylsulfoxide and without LPS stimulation were used as controls in all the experiments.

2.2. Cell viability assay

Chondrocytes were plated in 96-well plates at a density of 15,000 cells per well in phenol red-free medium and kept overnight. Cells were then treated with increasing doses of genistein (0, 25, 50, 100, and 200 μM). After 1 h, chondrocytes were treated with 1 μg/ml LPS for 24 h. The number of viable cells was determined as a function of metabolic activity using the dye resazurin according to the manufacturer's directions. Briefly, culture medium was removed, and 200 μl culture medium containing 10% resazurin (Sigma, Saint Louis, MO) was added to each well. Cells were incubated at 37°C in 5% CO_2 for 4 h. The absorbance was measured at reference wavelength of 690 nm and subtracted from the 600-nm measurement.

2.3. Measurements of COX-1 and COX-2 using Western blot analyses

Chondrocyte cultures (80% confluent) were washed with phosphate-buffered saline (PBS) twice. Nuclear and cytosolic fractions were extracted with nuclear extraction kit (Panomics, Redwood city, CA, USA). Equal amounts of protein (20 μg) of cytosolic fraction estimated by bicinchonic acid reagent (Sigma) were loaded onto 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene diflouride membrane (Millipore, Bedford, MA, USA) using a semidry blotting apparatus. Membranes were blocked with 1% Tris-buffered saline (TBS; 8 mM Tris HCl, 16 mM Tris-base, 150 mM NaCl) containing 5% skim milk for 1 h. After washing in TBS, blots were incubated with a 1:200 dilution of goat polyclonal COX-2 and rabbit polyclonal COX-1 (Santa Cruz Biotechnology, CA, USA) antibody overnight, respectively. This was followed by 2-h incubation with 1:2000 dilutions of goat polyclonal and rabbit polyclonal (Santa Cruz Biotechnology) secondary antibodies in blocking buffer. The protein bands were visualized using an Immun-Star HRP substrate kit (Bio-Rad laboratories, Hercules, CA, USA) according to manufacturer's instructions.

2.4. Measurement of NO release

Nitrite is a stable oxidant product of NO, and accounts for approximately half of the total of NO released by chondrocytes that accumulate as nitrite in culture medium. Hence, NO release was assessed by measuring nitrite levels in the culture supernatants collected after 24 h using Griess reagent (Promega, Madison, WI, USA). Sodium nitrite dissolved in tissue culture medium (0.1 μM–100 M) was used to create the standard curve for this assay. Samples (50 μl) and standard media were combined with 50 μl of sulfanilamide, incubated for 10 min at room temperature.

Then, 50 μl *N*-(1-naphthyl)ethylenediamine dihydrochloride solution was added to all wells and incubated for another 10 min. Absorbance was measured at 520–550 nm. Nitrite levels were determined using the standard curve generated for each plate.

2.5. Assessment of IL-1β

Interleukin-1β was measured in culture medium with enzyme-linked immunosorbent assay (ELISA) (PromoKine Bioscience Alive, Heidelberg, Germany), which is designed to measure free cytokines in cell culture supernatants. Standards (1000, 500, 125, 31.25, 7.81, and 0.0 pg/ml) and samples (100 μl) were added to microtiter plates precoated with monoclonal antibodies. Rabbit antihuman IL-1β polyclonal antibody was added into each well. After 3 h incubation at room temperature, plate was washed with PBS four times. Goat antirabbit-conjugated alkaline phosphatase (50 μl) was added into each well followed by 45-min incubation at room temperature. Plate was washed four times with buffer, and then 200 μl of color reagent was dispensed into each well. The reaction was stopped after 15 min of incubation at room temperature by adding 50 μl of stop solution into each well. Absorbance was read at 492 nm and values were reported as pg/ml.

2.6. Immunoassay for YKL-40

YKL-40 was measured by a sandwich enzyme immunoassay (EIA) kit (Metra, Quidel, San Diego, CA, USA) in a microtiter stripwell format using aliquots of cell culture supernatant. Cell culture media (20 μl) and standards (0.0, 50, 100, 200, and 300 ng/ml) were added to each streptavidin-coated well. Monoclonal anti-YKL-40 antibody conjugated to biotin binds to streptavidin on the strip and captures YKL-40 in standards and samples. After 1 h of incubation at room temperature, the plate was washed with a buffer containing sodium azide (0.05%). Polyclonal anti YKL-40 antibody conjugated to alkaline phosphatase (100 μl) was added to each well to bind to the captured YKL-40, followed by 1 h of incubation at room temperature and washing with buffer containing sodium azide (0.05%). Then, 100 μl of a diethanolamine and magnesium chloride solution containing sodium azide (0.05%) and *p*-nitrophenyl phosphate was added to each well and incubated at room temperature for 1 h. Bound enzyme activity was detected with *p*-nitrophenyl phosphate as substrate, and the reaction was stopped with addition of 100 μl of 1 mol/l NaOH to each well. The absorbance was read at 405 nm with a microplate reader and values were reported as ng/ml.

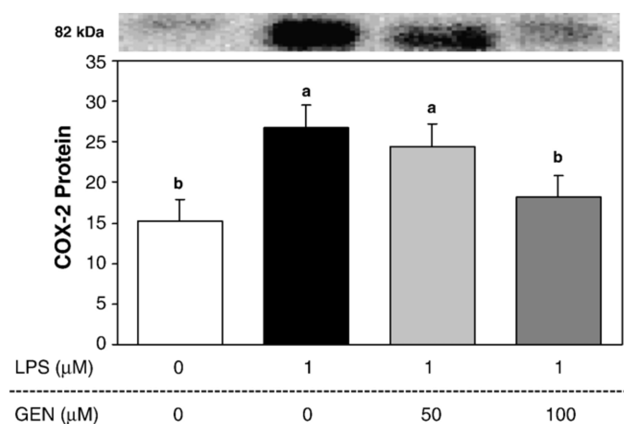

Fig. 2. COX-2 levels in cytosolic fraction of chondrocytes. Cytosolic protein extraction was separated by SDS-PAGE, and immunostain was performed with anti-COX-2 antibody. Bars represent mean±S.E., $n=3$ per treatment group. Bars with different letters are significantly different from each other ($P<.05$).

2.7. Statistical analysis

The data presented are the mean±S.E. To assess statistical significance, values were compared using analysis of variance (ANOVA). When ANOVA indicated statistical significance, Tukey's post hoc multiple comparisons test was used to determine which means were significantly

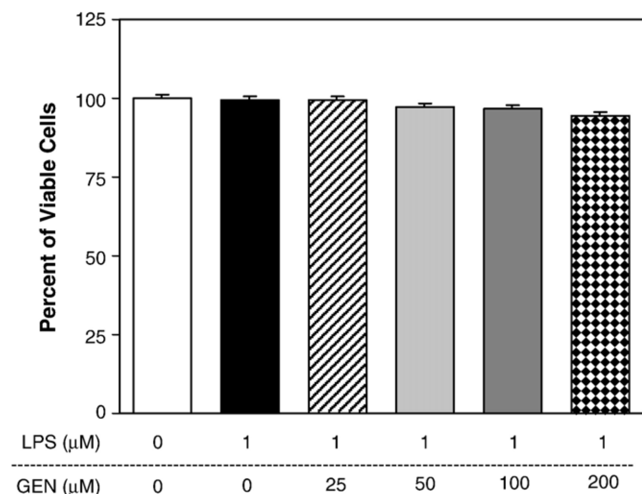

Fig. 1. Effects of LPS and gradient doses of genistein on viability of cultured human chondrocytes. Bars represent mean±S.E., $n=4$ per treatment group. GEN, genistein.

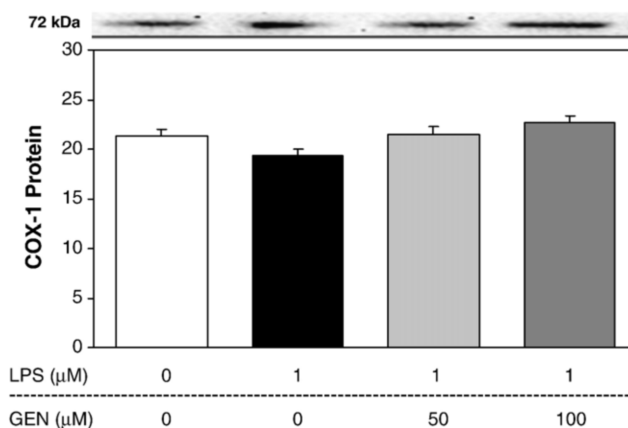

Fig. 3. COX-1 levels in cytosolic fraction of chondrocytes. Cytosolic protein extraction was separated by SDS-PAGE, and immunostain was performed with anti-COX-1 antibody. Bars represent mean±S.E., $n=3$ per treatment group.

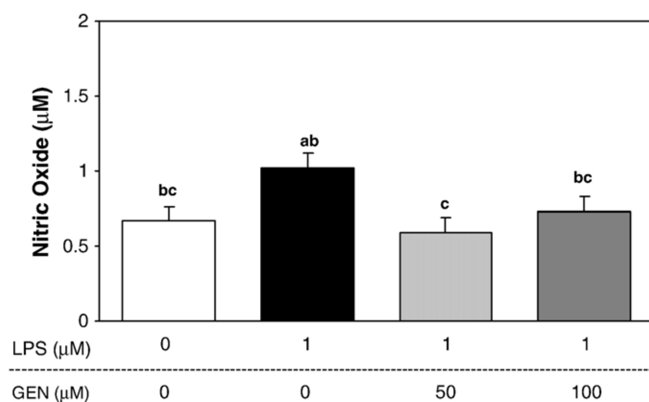

Fig. 4. NO level in culture supernatant, which was measured via Griess reagent. Bars represent mean±S.E., n=4 per treatment group. Bars with different letters are significantly different from each other (P<.05).

different. GraphPad software version 3.0 (San Diego, CA, USA) was used for all statistical analyses. Significance was accepted at $P \leq .05$.

3. Results

3.1. Cell viability

Treatment with LPS did not negatively affect the viability of chondrocytes compared to the control group. Combination of LPS (1 μg/ml) and genistein in doses of 25, 50, 100, and 200 μM did not alter cell viability of chondrocytes (Fig. 1).

3.2. Protein Level of COX-2 and COX-1

LPS significantly increased the COX-2 protein abundance in chondrocytes. Genistein at the dose of 100 μM significantly prevented this LPS-induced increase (P<.05) (Fig. 2). Genistein treatment had no effect on COX-1 protein level (Fig. 3).

3.3. Nitric oxide production

NO levels in cell culture supernatants increased as a result of LPS treatment (Fig. 4). Genistein in dose of 50 μM was able to reduce NO production significantly in comparison with LPS-treated control cells.

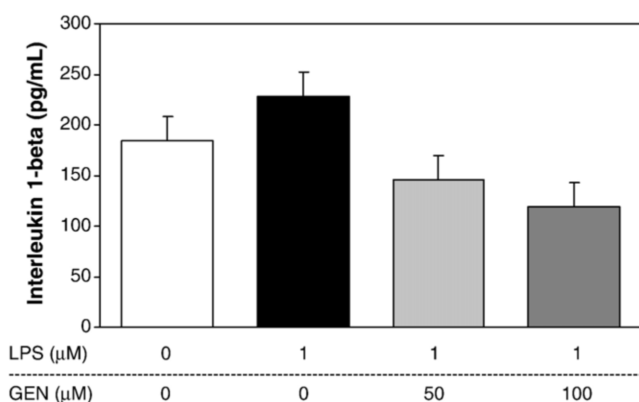

Fig. 5. IL-1β level in culture supernatant measured via ELISA kit. Bars represent mean±S.E., n=4 per treatment group.

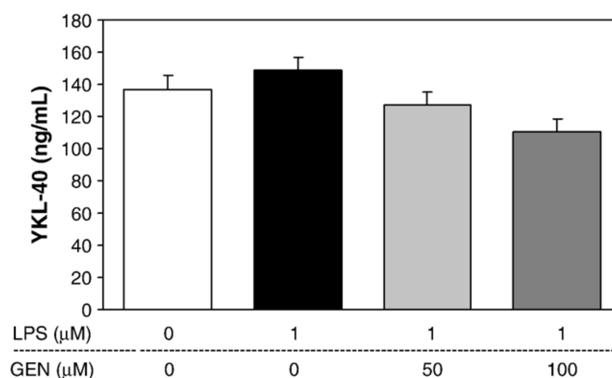

Fig. 6. YKL-40 level in culture supernatant which was measured via EIA kit. Bars represent mean±S.E., n=4 per treatment group.

3.4. Interleukin-I beta production

IL-1β mean values were numerically lower in genistein-treatment groups by 36.4% and 48%, respectively, for doses of 50 and 100 μmol/ml of genistein in comparison with LPS-treated group. Overall, there were no significant differences among the treatment groups (Fig. 5).

3.5. YKL-40 production

YKL-40, a marker of human cartilage glycoprotein degradation, increased in LPS-treated group. Both doses of genistein were able to suppress its levels in cell supernatant by 18.6% and 29.3%, respectively, compared to LPS-treated group (albeit not significantly) (Fig. 6).

4. Discussion

The findings of the present study indicate that genistein, a soy isoflavone, suppresses the production of proinflammatory molecules such as COX-2 and NO in LPS-induced chondrocytes, while it has no effect on COX-1 production. Isoflavones are thought to act similarly to estrogen, which is known [29,30] to exert anti-inflammatory effects. Soy isoflavones are considered natural SERMs [19] because of their conformational ability to bind to ERs, particularly the beta subtypes [31], in a manner similar to other SERMs such as raloxifene. Genistein suppressed the protein levels of COX-2; however, several discrete signaling pathways have been implicated in the genesis of COX-2 synthesis that are dependent on the stimulus imposed on cells. Several studies [19,32] have shown that COX-2 is partly controlled by nuclear factor kappa B (NF-κB). Largo et al. [32] have reported that inhibition of NF-κB activation was related to the down-regulation of the expression and synthesis of COX-2. COX is a critical proinflammatory enzyme that converts arachidonic acid to prostaglandins. Although prostaglandins have been implicated in the pain and inflammation associated with osteoarthritis [33], they may not fully explain neither joint inflammation nor OA symptoms. NSAIDs or COX

inhibitors have been extensively used in the treatment of OA [34,35]. It has been suggested that the anti-inflammatory action of NSAIDs are due to inhibition of COX-2, a cytokine-induced isoenzyme that mediates pain and inflammation, whereas the unwanted side effects such as the risk of significant injury to the upper gastrointestinal tract and lining of kidneys are due to inhibition of COX-1 [33,34]. Interestingly, in the present study genistein dose-dependently decreased the production of COX-2 protein level, while it had no such an effect on COX-1.

Other proinflammatory cytokines such as IL-1β and tumor necrosis factor α are believed to cause damage to cartilage by inducing matrix metalloproteinase (MMP) expression in chondrocytes [36–38]. Both cytokines activate synthesis and release of MMPs, which leads to matrix breakdown [39]. Elevated levels of IL-1β are also found in OA synovial fluid and gene expression of IL-1β has similarly been reported to be up-regulated in cartilage obtained from patients with knee OA [40].

While the findings of the present study indicate that genistein reduces LPS-induced IL-1β in chondrocytes in a trend similar to COX-2, these reductions cannot be due to cytotoxicity, as none of the doses of genistein reduced cell viability. IL-1β has been shown to induce production of NO in synovial cells and chondrocytes, which leads to increased vasodilation, permeability, and cartilage resorption in arthritic joints [41]. Additionally, NO inhibits proteoglycan synthesis, modulates the activity of metalloproteines, and induces apoptosis in human chondrocytes [42]. Although in other experimental models of inflammation and arthritis, NO has not been shown to either promote or prevent tissue injury [41], in chondcrocytes, NO has been demonstrated to inhibit collagen and proteoglycan synthesis [41] and induce apoptosis [43]. Hence, our finding that genistein suppresses the production of NO should be viewed as a positive finding. Nonetheless, further studies are needed to demonstrate that genistein reduces NO levels in animal models of OA and in humans with OA.

In the present study, YKL-40 level, a marker of cartilage degradation, was reduced in a similar fashion to COX-2 as a result of genistein treatment. Its serum levels are generally associated with increased cartilage breakdown [44], which is often triggered by inflammation. Immunohistochemical analysis of articular cartilage biopsy samples from the hip joint of patients with OA have shown positive staining for YKL-40 in chondrocytes. YKL-40 is synthesized by articular chondrocytes [28] of patients with OA or rheumatoid arthritis. The findings of a study by Volck et al. [45] indicated that YKL-40 expression in chondrocytes from normal cartilage was low or not detectable in comparison with patients with OA. In another study by Volck et al. [44], YKL-40 was detected in the inflamed synovial membrane, and the number of YKL-40 positive cells was associated with the degree of synovial inflammation.

The hypothesis of anti-inflammatory effect of genistein can be indirectly supported by observations of Volck et al. [44] who injected human arthritic joints with glucocorticoid and reported remission in joint inflammation followed by a decrease in serum YKL-40. In summary, the results of the present study show that genistein selectively decreases the production of LPS-induced COX-2 protein level in chondrocytes without affecting COX-1. If the results are shown to be reproducible, genistein can be of particular interest to individuals who suffer from chronic inflammatory conditions such as OA. As discussed earlier, there are no pharmaceutical agents that selectively can inhibit COX-2 production without having serious side effects. Therefore, if the results of our clinical study [20], using genistein as a dietary supplement instead of soy protein, are confirmed to be efficacious in alleviating symptoms and improving joint mobility in individual with OA in a longer and larger study, genistein can be an attractive and viable alternative therapy for treatment or prevention of OA. Furthermore, we can speculate from our soy clinical findings that genistein can be effective in improving the symptoms of OA in both men and women. Nonetheless, these need to be confirmed in future studies.

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