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Estrogen receptor-independent inhibition of tumor necrosis factor-α gene expression by phytoestrogen equol is mediated by blocking nuclear factor-κB activation in mouse macrophages

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ABSTRACT

Equol has been suggested to possess protective effects on bone. However, the underlying mechanism of osteoprotective effect of equol has not been fully understood. In the present study, we examined the effect of equol on tumor necrosis factor-α (TNF-α) gene expression to elucidate a possible mechanism by which equol exerts osteoprotective effect. In vivo administration of equol inhibited TNF-α production by peritoneal macrophages isolated from LPS-treated mice. Equol also dose-dependently inhibited TNF-α production and TNF-α mRNA expression in LPS-stimulated mouse macrophages. Pretreatment of cells with ICI 182.780, an estrogen receptor antagonist, had no effect on the inhibitory efficacy of equol on LPS-induced TNF-α production. Further study demonstrated that equol inhibited NF-κB DNA binding and NF-κB-dependent reporter gene expression in activated RAW 264.7 cells. Moreover, equol blocked degradation of IκBα and IκBβ and nuclear translocation of p65 subunit of NF-κB in activated RAW 264.7 cells. These results suggest that the inhibitory effect of equol on TNF-α expression is mediated, at least in part, by blocking NF-κB activation and the inhibition of TNF-α expression by equol might be involved in its osteoprotective effect.

1. Introduction

Osteoporosis is a skeletal disorder characterized by reduced bone strength predisposing to an increased risk of fracture. Approximately 30% of postmenopausal women are affected by this disease and this will become more prevalent as the world's population ages. Estrogen deficiency is a major contributing factor to the development of post-menopausal osteoporosis in women. In postmenopausal women, estrogen deficiency is associated with increased bone turnover and

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acceleration of bone loss, which lead to an increased susceptibility to bone fracture [1]. It is now recognized that one of the main mechanisms by which estrogen deficiency causes bone loss is a stimulation of osteoclast formation [2], a process enhanced by several inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Once released in the bone microenvironment, TNF-α stimulates osteoclast formation, in part by inducing the production of macrophage-colony stimulating factor by bone marrow stromal cells [3,4]. Kimble et al. demonstrated that the functional blocking of TNF-α prevents bone loss in ovariectomized mice [5]. It has been also shown that transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency [6]. Thus, these studies suggest that TNF-α is a key regulator of bone resorption in estrogen-deficient mice.

Phytoestrogens are polyphenolic non-steroidal isoflavones with estrogen-like activity and known to play an important role in the prevention of cancers, heart disease, postmenopausal symptom and osteoporosis [7–9]. Phytoestrogens have been shown to bind to two types of estrogen receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ) [10,11]. It is generally accepted that the diverse biological activity of phytoestrogens is due, in part, to their ability to act as estrogen receptor agonists and antagonists. However, several phytoestrogens have been reported to exert other non-hormonal effects, including inhibition of tyrosine kinases [12], DNA topoisomerase I and II [13], and anti-angiogenesis [14] and antioxidant activity [15]. Equol [7-hydroxy-3-(4′-hydroxyphenyl)-chroman] is a product of intestinal bacterial metabolism of dietary isoflavone, daidzein, and it also possesses estrogenic property and an affinity for both ERα and ERβ [16]. Equol was shown to have superior antioxidant activity compared with other phytoestrogens [17] and also inhibit lipoprotein oxidation in vitro [18].

Nuclear factor-κB (NF-κB) is known to play a critical role in the development of inflammatory response by upregulating the expression of many inflammatory mediators [19]. TNF-α gene expression was also known to be regulated by NF-κB transcriptional activity [20]. NF-κB is a dimer of members of the Rel family proteins, including p65, c-Rel and p50. The activity of NF-κB is primarily controlled at the post-transcriptional level [21]. In unstimulated cells, NF-κB exists in an inactive state in the cytoplasm complexed with the inhibitory protein called inhibitory factor-κB (IκB). Upon activation, IκB undergoes phosphorylation and degradation, and the NF-κB heterodimer is translocated into the nucleus where it binds to DNA and activates transcription [22]. De novo synthesis of new IκB proteins occurs after its phosphorylation and degradation by IκB kinase and then IκB enters the nucleus, dissociates NF-κB from DNA, and again inactivate NF-κB [23].

Because LPS is a general inducer of macrophage activation and a potent activator of NF-κB activity and TNF-α production in macrophages, we used LPS-induced macrophage model system to examine the effect of equol on TNF-α gene expression. In the present study, we investigated the effect of equol on TNF-α production to understand the underlying mechanism responsible for the osteoprotective effect of dietary phytoestrogens. We also assessed the mechanism involved in the inhibitory effect of equol on TNF-α gene expression. Our results suggest that equol inhibits TNF-α gene expression in an estrogen receptor-independent manner in mouse macrophages and this is mediated by blocking NF-κB activation.

2. Materials and methods

2.1. Chemicals, animals, and cell culture

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. Equol was dissolved in DMSO and freshly diluted in culture media for all in vitro experiments. Virus-free female BDF1 mice were purchased from Dae Han Laboratory Animal Research Center Co., Ltd. (Chungbuk, Korea) and cared for as described previously [24]. For in vivo administration, equol was dissolved in 5% Tween-80 (v/v). Equol was administrated i.p. 1 h before LPS (200 μg/kg, i.p.) treatment. The peritoneal macrophages and RAW 264.7 cells (ATCC TIB71) were grown in RPMI 1640 and Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), respectively, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2 humidified air. Peritoneal macrophages were harvested by sterile peritoneal lavage using PBS, washed, resuspended in culture medium, and plated at 2×10^6 cells/ml. Nonadherent cells were removed by repeated washing after 2-h incubation at 37 °C.

2.2. ELISA

Mouse peritoneal macrophages and RAW 264.7 cells were plated at 2×10^6 and 5×10^5 cells/ml, respectively, and stimulated with LPS (200 ng/ml) for 6 h in the presence or absence of equol (0.3, 1, 3 or 10 μg/ml) or ICI 182.780. Culture supernatants were taken and the concentration of TNF-α was determined by sandwich immunoassays using a protocol supplied by R&D Systems (Minneapolis, MN, USA).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

The expressions of the mRNA transcripts of TNF-α (forward primer: 5′-CCTGTAGCCCACGTCGTAGC-3′, reverse primer: 5′-TTGACCTCAGCGCTGAGTTG-3′), and β-actin (forward primer: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, reverse primer: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′) were evaluated by RT-PCR as described previously [25] with slight modifications. Briefly, total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously [26]. Equal amounts of RNA were reverse transcribed into cDNA using oligo$(dT)_{15}$ primers. Samples were heated to 94 °C for 5 min and cycled 30 times at, 94 °C for 30 s, and 56 °C for 30 s, and 72 °C for 45 s, and this was followed by an additional extension step at 72 °C for 5 min. For quantitation of TNF-α cDNA, we generated an internal standard (IS). PCR products for IS which can be amplified using TNF-α primer listed above were generated (IS forward primer: 5′-CCTGTAGCCCACGTCGTAGCATGGTGGGAATGGGTCAGAAGGAC-3′, IS reverse primer: 5′-TTGACCTCAGCGCTGAGTTGCTCTTTGATGTCACGCA-

CGATTTC-3′) and subcloned into pGEM T Easy vector (Promega Corporation, Madison, WI, USA). IS PCR products (552 bp) can be distinguished from wild type PCR products (374 bp). Standard curve for competitive PCR was generated by performing a PCR reaction using cDNA from LPS-treated sample and serially diluted IS and analyzing their relative band intensities. Finally, 10^{-13} μM IS was used to quantitate the number of TNF-α cDNA of samples. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME, USA) and followed by ethidium bromide staining and photography. Band intensities were quantified using Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously [25]. The protein content of the nuclear extracts was determined using a Bio-Rad protein assay kit according to the manufacturer's instruction (Amersham Biosciences UK, Ltd.). The oligonucleotide sequence for NF-κB was 5′-GATCTCAGAGGGGACTTTCCG-AGAGA-3′ [27]. Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI-dC) and a ^{32}P-labeled DNA probe, and DNA binding activity was analyzed using a 5% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography. The specificity of binding was examined by competition with an unlabeled oligonucleotide.

2.5. Transient transfection and CAT reporter gene assay

p(NF-κB)$_3$CAT plasmid has been described previously [28]. Transient transfection was performed using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA). Eighteen hours after transfection, cells were harvested, plated at 5×10^5 cells/ml, treated with the indicated concentrations of equol (0.3, 1, 3 or 10 μg/ml) for 1 h before the treatment of LPS (200 ng/ml), harvested 24 h after LPS treatment, and lysed. The CAT enzyme expression levels were determined using a CAT ELISA kit according to the manufacturer's instruction (Roche Applied Science, Mannheim, Germany).

2.6. Western immunoblot analysis

A 20 μg of cytosolic extract (for IκBα, IκBβ, IKKα and p65) and nuclear extract (for p65) were separated by 10% SDS-polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose membrane (Amersham Bioscience UK, Ltd., Little Chalfont, Buckinghamshire, UK). The membranes were preincubated for 1 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% Tween 20 and 5% non-fat milk. The nitrocellulose membranes were then incubated with specific antibodies against IκBα, IκBβ (Santa Cruz Biotechnology, Waltham, MA, USA), IKKα or p65 (Cell signaling Technology, Beverly, MA, USA). Immunoreactive bands were then detected by incubating with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Biosciences UK, Ltd.).

2.7. Statistical analysis

Means ± S.D. were determined for each treatment group in each experiment. Data were analyzed by ANOVA, and the Student's *t*-test was used for multiple comparisons. The criterion for statistical significance was set at $p < 0.01$.

3. Results

3.1. Effect of in vivo exposure of equol on TNF-α production in mouse peritoneal macrophages

To investigate the effect of in vivo exposure of equol on TNF-α production by peritoneal macrophages, we treated female BDF1 mice with LPS and/or equol and measured TNF-α production by peritoneal macrophages isolated from these mice. The administration of LPS (200 μg/kg, i.p.) to female BDF1 mice caused a significant increase in the production of TNF-α by peritoneal macrophages. However, treatment with equol (20 and 50 mg/kg, i.p., 1 h before LPS treatment) inhibited LPS-induced increase in TNF-α production in a dose-dependent manner (Fig. 1).

3.2. Effect of equol on TNF-α production in isolated mouse peritoneal macrophages and RAW 264.7 cells

To further confirm the effect of equol on TNF-α production in in vitro cultures, we examined the effect of equol on TNF-α

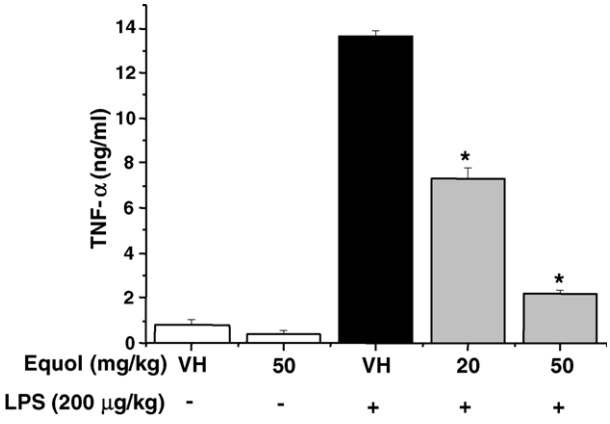

Fig. 1 – Effect of in vivo exposure of equol on the TNF-α production by peritoneal macrophages isolated from LPS-treated mice. Different groups ($n = 3$) of animals were treated with vehicle (5% Tween 80 v/v), equol (50 mg/kg at 1 h before LPS treatment, i.p.), LPS (200 μg/kg, i.p.) + vehicle (5% Tween 80 v/v), and LPS (200 μg/kg, i.p.) + equol (20 and 50 mg/kg at 1 h before LPS treatment, i.p.). Peritoneal macrophages were isolated 6 h after LPS treatment and cultured for 24 h in the absence of LPS and equol. The culture supernatants were subsequently isolated and analyzed for TNF-α production as described in Section 2. Each value shows the mean ± S.D. of triplicate determinations. Significance was determined using Student's *t*-test vs. the LPS-treated group (*$p < 0.01$).

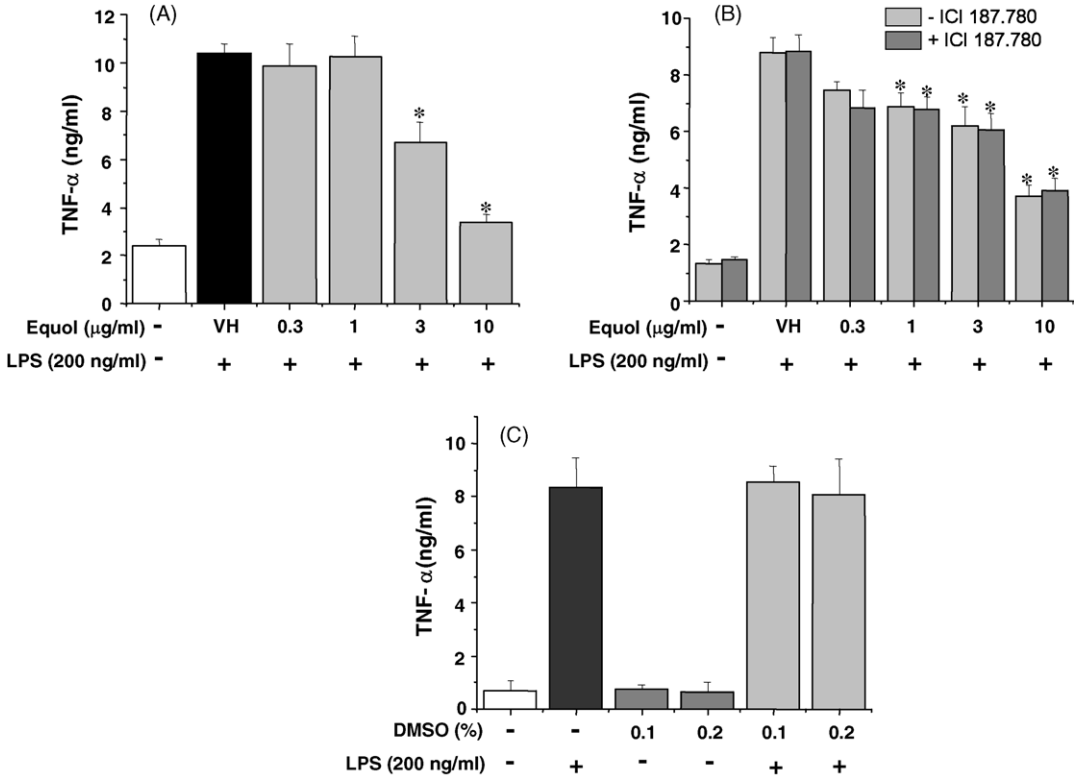

Fig. 2 – Inhibition of TNF-α production by equol in LPS-stimulated peritoneal macrophages and RAW 264.7 cells. (A) Peritoneal adherent cells were pretreated with vehicle (0.1% DMSO) or indicated concentrations of equol for 1 h before being incubated with LPS (200 ng/ml) for 6 h. (B) RAW 264.7 cells were pretreated with ICI 182.780 (100 nM) for 1 h and treated with the indicated concentrations of equol for 1 h before being incubated with LPS (200 ng/ml) for 6 h. (C) RAW 264.7 cells were pretreated with the indicated concentrations of DMSO before being incubated with LPS (200 ng/ml) for 6 h. The culture supernatants were subsequently isolated and analyzed for TNF-α production. Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using Student's *t*-test vs. the LPS-treated group (*$p < 0.01$).

production in isolated peritoneal macrophages and RAW 264.7 cells, a murine macrophage-like cell line that can be stimulated to acquire the osteoclastic phenotype. As shown in Fig. 2A, LPS (200 ng/ml) alone caused 4.3-fold induction of TNF-α production in peritoneal macrophages, but this induction was significantly suppressed by equol. In RAW 264.7 cells, LPS evoked a 6.5-fold induction of TNF-α production versus the untreated control, and this was also inhibited by equol in a dose-dependent manner (Fig. 2B). As described previously, estrogen is known to downregulate TNF-α production [4]. Because equol is also known to have estrogenic activity and RAW 264.7 cells are known to express estrogen receptors [4], we examined whether the inhibitory effect of equol on TNF-α production is estrogen receptor-dependent or not. As shown in Fig. 2B, pretreatment of RAW 264.7 cells with ICI 182.780, an estrogen receptor antagonist, had no effect on the inhibitory action of equol on LPS-induced TNF-α production. We also confirmed that the concentrations of DMSO used in this study had no significant effect on the TNF-α production in the presence or absence of LPS (Fig. 2C). The concentration of equol and the duration of equol treatment had no significant effect on the viability of isolated peritoneal macrophages and RAW 264.7 cells (data not shown).

3.3. *Effect of equol on TNF-α mRNA expression in LPS-stimulated RAW 264.7 cells*

To investigate whether the inhibitory effect of equol on TNF-α production is due to the reduced gene expression of TNF-α, we assessed the effect of equol on TNF-α mRNA expression using RT-PCR. As shown in Fig. 3A, equol inhibited LPS-induced expression of TNF-α mRNA in a dose-dependent manner. In contrast to TNF-α, the level of β-actin mRNA expression remained the same under these conditions. We also confirmed the effect of equol on LPS-induced TNF-α mRNA expression using competitive RT-PCR. Fig. 3B and C shows that the LPS-induced expression of TNF-α mRNA is significantly suppressed by the treatment of 3 and 10 μg/ml equol.

3.4. *Effect of equol on LPS-induced NF-κB DNA binding and transcriptional activity in RAW 264.7 cells*

NF-κB is an important transcriptional regulator involved in TNF-α gene expression [29]. To assess if the inhibitory effect of equol on TNF-α expression is mediated via NF-κB, we examined the effect of equol on NF-κB activity using electrophoretic mobility shift assay (EMSA) and chloramphenicol acetyltrans-

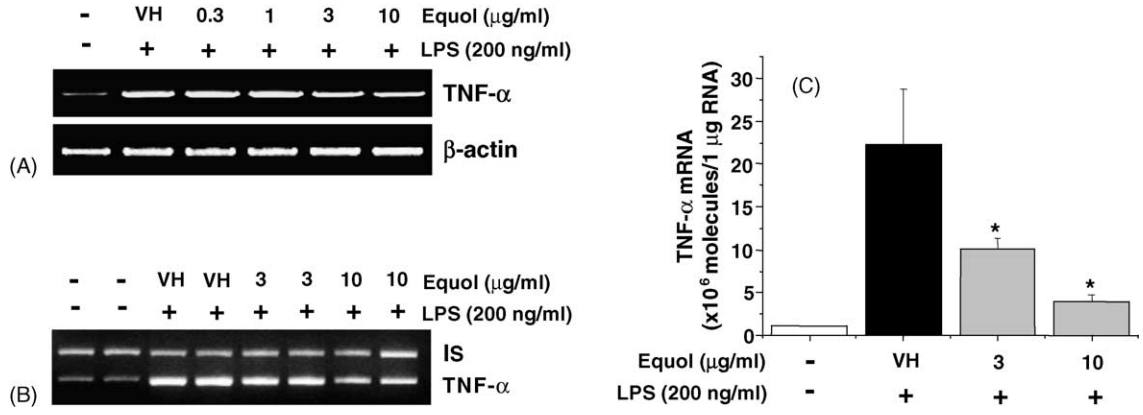

Fig. 3 – Inhibition of TNF-α mRNA expression by equol in LPS-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with vehicle (0.1% DMSO) or indicated concentrations of equol for 1 h before being incubated with LPS (200 ng/ml) for 6 h. Total RNAs were isolated and TNF-α mRNA expression was determined by RT-PCR as described in Section 2. (B) Competitive RT-PCR was performed and (C) the number of TNF-α cDNA generated from 1 μg total RNA was quantitated as described in Section 2. Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using Student's t-test vs. the LPS-treated group (*$p < 0.01$).

ferase (CAT) assay. Treatment of RAW 264.7 cells with LPS (200 ng/ml) caused a significant increase in the DNA binding activity of NF-κB within 1 h (Fig. 4A). In the presence of equol, LPS-induced NF-κB DNA binding was markedly suppressed in a concentration dependent manner (Fig. 4A). Supershift assays showed that p65 and p50 are main component of LPS-induced NF-κB complex in RAW 264.7 cells (Fig. 4B). The specificity of binding was also examined using unlabeled wild type and mutant NF-κB probe (Fig. 4B). To further confirm the inhibitory effect of equol on NF-κB, we examined the effect of equol on the

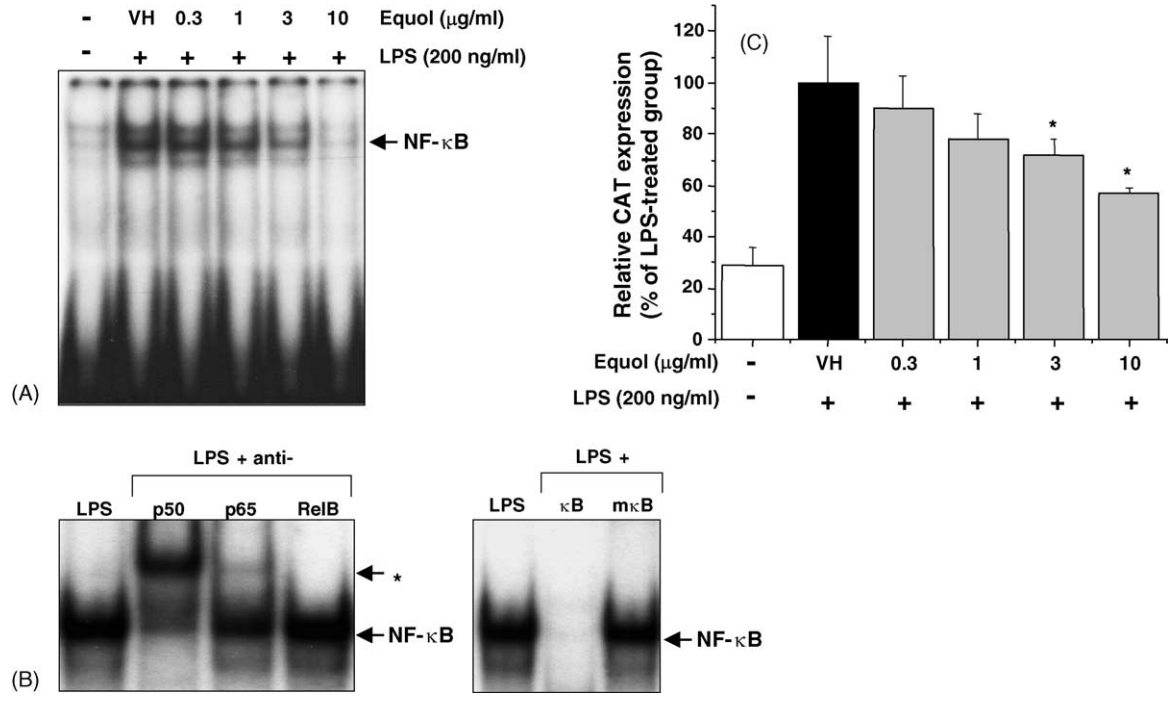

Fig. 4 – Effect of equol on LPS-induced DNA binding of NF-κB and NF-κB-dependent reporter gene expression in RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with vehicle (0.1% DMSO) or indicated concentrations of equol for 1 h before being incubated with LPS (200 ng/ml) for 1 h. Nuclear extracts were then prepared and DNA bindings of NF-κB were determined by electrophoretic mobility shift assay. (B) Nuclear extracts isolated from LPS-treated group were preincubated with the indicated antibodies, unlabeled NF-κB probe and mutant NF-κB probe before being incubated with ^{32}P-labeled NF-κB probe, and electrophoretic mobility shift assay was performed. (*) Supershifted NF-κB. (C) RAW 264.7 cells were transiently transfected with p(NF-κB)$_3$-CAT and the expression of CAT enzyme was analyzed by ELISA as described in Section 2. Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using Student's t-test vs. the control group (*$p < 0.01$).

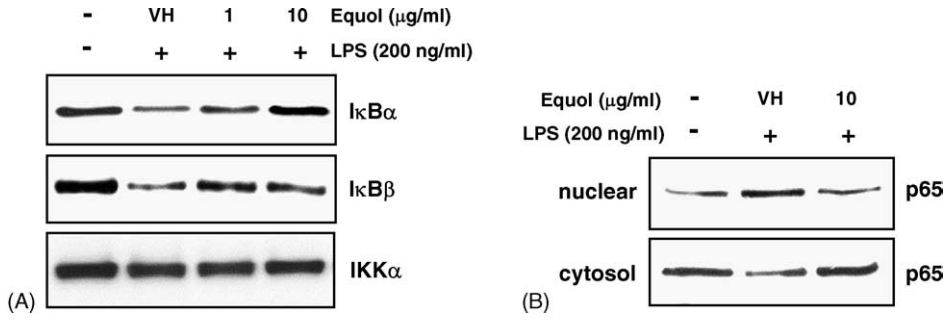

Fig. 5 – Effect of equol on the LPS-induced degradation of IκBα and IκBβ and on the nuclear translocation of the p65 subunit in RAW 264.7 cells. (A) Cells were pretreated with the indicated concentrations of equol for 1 h, incubated with LPS (200 ng/ml) for 30 min, and then assayed for IκBα, IκBβ and IKKα in cytosolic fractions by Western immunoblot analysis as described in Section 2. (B) The nuclear translocation of the p65 subunit was determined by Western immunoblot analysis using nuclear and cytosolic fractions.

NF-κB-dependent reporter gene expression. We performed reporter gene assay using p(NF-κB)$_3$CAT plasmid, which was generated as described previously [28]. RAW 264.7 cells transfected with p(NF-κB)$_3$CAT plasmid were stimulated with 200 ng/ml LPS in the presence or absence of equol. CAT enzyme expression was increased 7.5-fold after stimulation with LPS for 24 h, and the treatment of equol significantly reduced the LPS-induced increase in NF-κB-dependent CAT enzyme expression in a concentration dependent manner (Fig. 4C).

3.5. *Effect of equol on IκB degradation and p65 nuclear translocation in LPS-stimulated RAW 264.7 cells*

The activation of NF-κB occurs via the nuclear translocation of Rel family proteins, an event preceded by the phosphorylation and degradation of IκBs by IKK. Therefore, we investigated whether the inhibition of NF-κB activity by equol is mediated through the modulation of IκB degradation and p65 nuclear translocation. First, the cytoplasmic levels of IκBα and IκBβ were determined by Western immunoblot analysis. The degradation of IκBα and IκBβ was detected 30 min after LPS (200 ng/ml) treatment, and the degradation of these proteins was blocked by equol treatment (Fig. 5A). The expression level of IKKα, one of the major kinase that phosphorylates IκBα and IκBβ, was not affected by treatment with LPS or equol (Fig. 5A). Next, we examined the effect of equol on the nuclear translocation of the p65 subunit of NF-κB. As shown in Fig. 5B, the level of p65 nuclear translocation was increased in LPS-treated group, and in the presence of equol, LPS failed to induce nuclear translocation of p65 subunit. In contrast, the cytosolic level of p65 was diminished after LPS treatment, and this was blocked by equol treatment (Fig. 5B).

4. Discussion

As mentioned earlier, cytokines, such as TNF-α and IL-1β, produced by monocyte–macrophage lineage in bone microenvironment, are potentially important local regulators of bone turnover. It is well known that TNF-α is a potent stimulator of bone resorption and the regulation of TNF-α gene expression by estrogen is important for the development of osteoporosis [4]. Although TNF-α is just one of several cytokines that participate in pathophysiology of osteoporosis, evidences suggest that TNF-α performs a central role in the occurrence of osteoporosis [4,30,31]. At physiological concentrations, estrogen can either increase or decrease cytokine gene expression depending on species as well as stimulus [32–34]. Although there are many reports demonstrating a complex regulation of cytokine expression by estrogen, considerable evidences support that estrogen prevents bone loss by blocking the production of cytokines, including TNF-α and IL-1β, in the bone microenvironment [5,6,35,36]. Moreover, it has been reported that increased production of proinflammatory cytokines were observed after natural or surgical menopause [37–39], demonstrating the inhibitory effect of estrogen on the expression of proinflammatory cytokines. In the present study, we showed that equol exerts an inhibitory effect on TNF-α gene expression in macrophages in both in vivo and in vitro conditions. Our results suggest that equol may also exert its osteoprotective effect, at least in part, by inhibiting TNF-α production.

There have been several reports describing inhibitory mechanism by which estrogen inhibits TNF-α gene expression. First, estrogen is proposed to suppress expansion of TNF-producing clones of T cells [40,41]. In the second, it was suggested that estrogen directly suppressed transcription of the TNF-α gene [4]. Srivastava et al. reported that estrogen decreases TNF-α gene expression by blocking c-jun NH_2-terminal kinase (JNK) activity and the resulting production of c-jun and junD in RAW 264.7 cells [4]. In contrast to this result, equol had no effect on JNK activation in LPS-stimulated RAW 264.7 cells (unpublished data), suggesting that the mode of action of equol and estrogen is not exactly same. Indeed, although equol was shown to have strong estrogen receptor binding affinity, our data clearly shows that the inhibitory effect of equol on LPS-induced TNF-α production is independent of estrogen receptor. The NF-κB family of transcription factors are central mediators in the regulation of a variety of immune responses. The transcriptional regulation of TNF-α gene is also known to be regulated by NF-κB [42]. In the present study, we demonstrated that equol inhibits LPS-induced TNF-α gene expression, at least in part, by blocking NF-κB DNA binding and transcriptional activity. In agreement with our result, environmental estrogens, bisphenol A and 4-nonyl-

phenol, was also shown to suppress inducible nitric oxide synthase and TNF-α expression via inhibition of NF-κB activity [43,44]. We also demonstrated that equol inhibits LPS-induced degradation of IκBα and IκBβ, and the nuclear translocation of p65 subunit in RAW 264.7 cells. NF-κB-dependent reporter gene expression was also suppressed by equol treatment in LPS-stimulated RAW 264.7 cells. However, the inhibition of NF-κB-dependent reporter gene activity by equol is weaker than that of DNA binding activity. This may be because equol has weak or no effect on the transactivation potential of p65. Therefore, although low levels of NF-κB proteins are present in nucleus in equol-treated cells, they can drive NF-κB-dependent gene expression better than NF-κB proteins in unstimulated cells. It is well known that reactive oxygen species (ROS) pathway regulates the NF-κB activation [45]. Because equol is known to have strong free radical scavenging and antioxidant activities [46], it is assumed that the inhibition of NF-κB by equol may be mediated by the regulation of ROS pathway.

High phytoestrogen intake among Asian women has been thought to explain the low risk of bone fractures in these populations. A lot of studies using rat model of postmenopausal osteoporosis showed a significant bone-sparing effect of the phytoestrogens [47,48]. Nikander et al. also reported the protective effect of phytoestrogens on bone loss in postmenopausal women [49]. Picherit and coworkers demonstrated that daidzein, an isoflavone abundant in soybean extract, is more efficient than genistein in preventing ovariectomy-induced bone loss in rats even though daidzein has poor binding affinity to estrogen receptors and weak transcriptional activity compared to genistein [16,48]. These reports indicate that the estrogenicity of these compounds is not the only factor responsible for their osteoprotective activity. Although equol is known as an end product of intestinal bacterial metabolism of daidzein, it is not produced in all healthy adults after soy or daidzein uptake. The ability to produce equol is known to be dependent on the ''bateriotype'' of individuals [17]. Interestingly, Lydeking-Olsen et al. demonstrated that women who are ''equol producers'' experience greater protective effect on bone after uptake of isoflavone-rich soymilk [50], indicating that osteoprotective effect of soybean isoflavones and daidzein may be mediated by their conversion to equol. Indeed, equol was shown to potently inhibit bone loss in ovariectomized mice [51]. These reports explain why daidzein can exert stronger osteoprotective effect than genistein regardless of its weak estrogenic activity. However, in spite of its strong estrogen receptor binding affinity [16], equol was reported to inhibit bone loss without estrogenic activity in the reproductive organs of ovariectomized mice [51], suggesting an estrogen receptor-independent osteoprotective effect of equol. As mentioned earlier, the inhibition of TNF-α production by equol was estrogen receptor-independent. Considering the importance of TNF-α in the regulation of bone turnover, our results suggest that the inhibitory effect of equol on TNF-α production may be involved in the estrogen receptor-independent osteoprotective effect of equol. Collectively, it is assumed that phytoestrogen exerts its osteoprotective effect via both estrogen receptor-dependent and estrogen receptor-independent mechanisms. The latter mechanisms might be mediated by conversion of phytoestrogens to equol and subsequent inhibition of TNF-α production.

Taken together, our results demonstrate that equol inhibits LPS-induced TNF-α gene expression in macrophages and that these effects are mediated, at least in part, by inhibiting NF-κB activity. The results presented in this report suggest one of the possible mechanisms responsible for osteoprotective and bone sparing effect of dietary phytoestrogens. Our results also suggest a direct application of equol as alternative or supplement of hormone replacement therapy, especially for ''equol non-producers''.

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