약학석사학위논문

Soy isoflavone daidzein에 의한 레티노이드 수용체 활성화 연구

Activation of retinoic acid receptors by soy isoflavone daidzein

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오 현 정

ABSTRACT

Activation of retinoic acid receptors by soy isoflavone daidzein

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Retinoids modulate keratinocyte proliferation, differentiation, collagen synthesis and degradation, inflammatory response, and reduce sebum production in the skin. They have been used as therapeutic agents for diverse skin diseases such as aged skin, acne and skin cancer. Retinoids actions are mediated by retinoic acid receptors (RARs). Among the three isotypes, RARα,β and ɣ, RARα and RARɣ are expressed and regulate retinoid target genes in the skin. Here, we found that extracts of two different soy beans, *Glycine max* and *Rhynchosia Nulubilis* increased transcriptional activity of RARα and RARɣ. Further fractionation revealed that among the fractions, ethyl acetate fractions up-regulated transcriptional activity of RARs. HPLC analysis of the extracts and ethyl acetate fractions showed that daidzin and genistin are the major constituents of the fractions.

Daidzin and its aglycone form, daidzein induce RAR transcriptional activity in dose-dependent manner. Genistin and genistein also up-regulated RAR transcriptional activity but their effects were weaker than that of daidzin and daidzein. Time resolved-fluorescence resonance energy transfer (TR-FRET) analysis demonstrated that daidzein bound both RARα and RARɣ ligand binding domains and recruited co-activators. Daidzein increased RAR target genes, RARα and RARɣ mRNA in time dependent manner in the keratinocyte, HaCaT. ChIP analysis showed that daidein treatment enhanced recruitment of RARα, RARɣ, and p300 on functional RARE in the RARɣ2 promoter. Protein levels of RARα and RARɣ were reduced by daidzein, similar to the reduction by all-trans retinoic acid. Matrix metallopeptidase-9, which degrades collagen and enhances skin aging, was up-regulated by TNFα treatment, and its mRNA level and gelatinolytic activity were reduced by daidzein. Together, these results suggest that daidzein may be a functional ligand of retinoic acid receptors and it could be a therapeutic candidate for skin diseases as an alternative of retinoid. Also, these findings could explain a new mechanism of anti-skin aging effects of daidzein

key Words: skin, retinoic acid receptor, ligand, daidzein

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Ⅰ. INTRODUCTION

Vitamin A and its derivatives referred to as retinoids are non-steroid hormones which play a important role in skin physiology (Roos et al., 1998). Retinoids regulate keratinocyte proliferation and differentiation, immune system, and repress sebum production. Topical and systemic retinoids treatments have been used as therapeutic agents for diverse skin diseases, including psoriasis, skin cancer, acne, and photoaging (Thacher et al., 2000). Retinoids functions are mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which have three different isoforms (α, β and γ). When ligands bind to RAR and RXR, they are activated, dissociate from corepressors and recruit coactivators to induce transcription of genes that contain retinoic acid response element (RARE) in their promoter regions (Bastien and Rochette-egly, 2004). The expression of the retinoic acid receptors is tissue specific. In case of human skin, RARα and RARγ are expressed in keratinocytes and fibroblasts which are major cell types of epidermis and dermis, and mediate retinoic acid actions (Redfern and Todd, 1992).

Although retinoids are potent therapeutic agents of diverse skin diseases, they have limitations in clinical utility because of their side effects such as skin irritation. Many efforts have been made to find retinoid derivatives which have not only the same efficacy as retinoids but also reduced skin irritation properties (Kambayashi et

al., 2005, Kim et al., 2008, Kim et al., 2010). In addition to retinoid derivatives, new drug design strategies have been made to find natural compounds that have chemical structures distinct from retinoic acid but activate retinoic acid receptor. For instance, lycopene which is a lipophilic carotenoid and is commonly found in tomatoes and its metabolites such as apo-10'-lypopenic acid activate RAR transcriptional activity (Aydemir et al., 2012, Gouranton et al., 2011). Also, recent research showed that the marine product lufariellolide, a sesterterpene, which is from hexane extract of *Lufariella* sp. and *Fascaply synopsi,* may act as a potent RAR ligand, regulating RAR related genes and inhibiting cancer cell growth (Wang et al., 2012).

Soy extracts contain soy isoflavones including genistein, daidzein and glycitein, which have been known to exhibit beneficial physiological functions such as anti-oxidant, anti-inflammatory response, and prevention of cardiovascular disease and osteoporosis. (Dalais et al., 2003, Kao and Chen, 2006, Kao et al., 2007). In the skin, topical application of soy isoflavones stimulates hyaluronic acid production, promotes wound healing and exerts anti-photoaging effect (Miyazaki et al., 2002, Emmerson et al., 2010, Huang et al., 2010). On the basis of their structural similarity with estrogen, isoflavones have been called phytoestrgens which are able to bind estrogen receptors and exert estrogenic or anti-estrogenic effect depend on estrogen concentration (pilsakova et al., 2010). It is reported that isoflavones act as ligands of not only estrogen receptors but also other nuclear receptors including peroxisome proliferator activated receptors

(PPARs) and estrogen-related receptors (ERRs) (Ricketts et al., 2005, Suetsugi et al., 2003). In this study, we demonstrated that daidzein, one of soy isoflavones, acts as an agonistic ligand of retinoic acid receptor and regulates retinoic acid related genes in the keratinocyte, suggesting the novel mechanism of soy isoflavones actions in the skin.

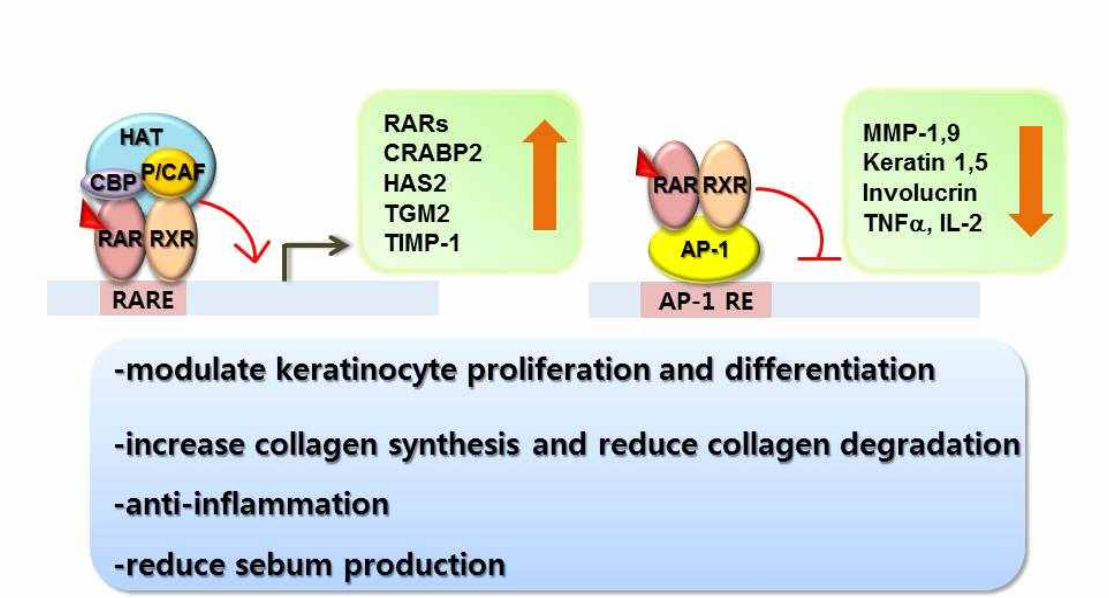


Fig1. Functions of ligand-activated retinoic acid receptor in the skin.

Retinoids functions are mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which have three different isoforms (α, β and γ). When ligands bind to RAR and RXR, they are activated to induce transcription of genes that contain retinoic acid response element (RARE) in their promoter regions or to repress AP-1 signaling. (Bastien and Rochette-egly, 2004). Retinoids regulate keratinocyte proliferation and differentiation, immune system, and repress sebum production in the skin.(Thacher et al., 2000).

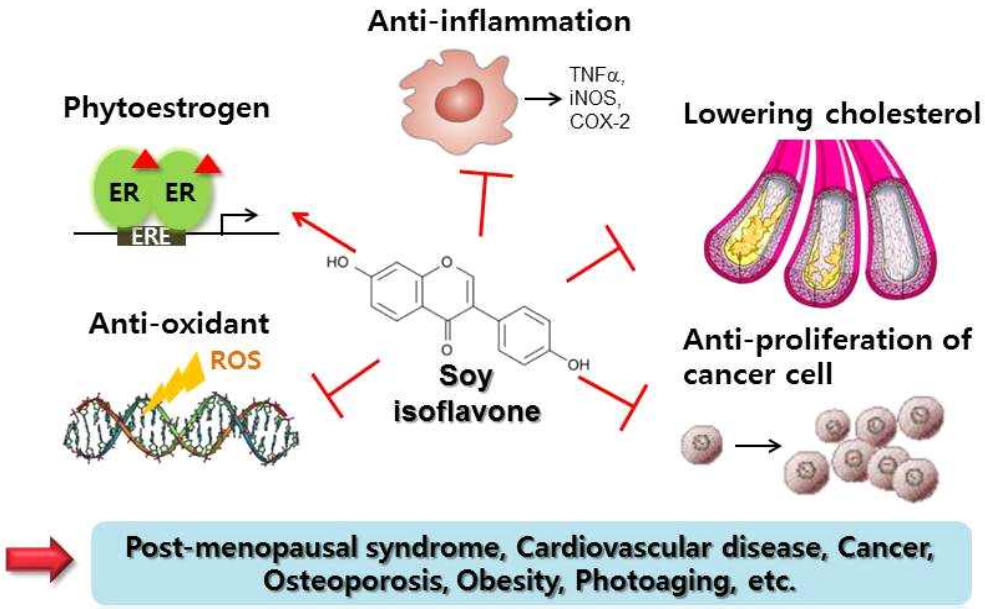


Fig2. Physiological functions of soy isoflavones.

Soy isoflavones are phytoestrogens which exhibit beneficial physiological functions such as anti-oxidant, anti-inflammatory response, and prevention of cardiovascular disease and osteoporosis. (Dalais et al., 2003, Kao and Chen, 2006, Kao et al., 2007).

Ⅱ. PURPOSE OF THE STUDY

Retinoids play critical roles in skin physiology. On the basis of their functions, retinoids are used as diverse skin diseases including skin aging, acne, skin cancer, psoriasis (Thacher et al. 2000). Retinoids actions are mediated by retionic acid receptors (RAR). In the skin, RARα and RARɣ are expressed and mediate retinoids functions. In spite of their efficacy, application of retinoids is limited due to their side effects such as skin irritation. Many efforts have been made to find retinoids alternatives from natural compounds that activate RARs but have less side effects.

In this study, we tried to find RAR activators from natural herbal extracts, and to identify these compounds could act as RAR ligands and exert retinoids-like actions in the keratinocyte.

Ⅲ. MATERIALS AND METHODS

1. Cell culture and reagents

CV-1, a green monkey kidney cell line (ATCC CCL 70), and HaCaT, a spontaneously transformed human keratinocyte cell line (Boukamp et al., 1988), were obtained from the American Type Culture Collection and the Cell Lines Service (Germany), respectively. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humid atmosphere of 5% CO2. All-trans retinoic acid (ATRA), daidzin, daidzein, genistin, and genistein were purchased from Sigma-aldrich (St Louis, MO).

2. Reporter gene assay

A modified calcium phosphate precipitation procedure was used for transient transfection (Kim et al., 2004). Briefly, CV-1 cells were seeded in a 24-well plate at $4*10^4$ cells per well and transfected with DNA mixtures (0.5 μg/well) containing reporter plasmid, ERE-Luc (0.1 μg), the receptor expression plasmid, pECE-ER-RARα or pECE-ER-RARɣ (0.05 μg), β-galactosidase (β-gal) expression vector (0.2 μg) with carrier DNA (pBluescript). The cells were treated for 24h with DMSO, all-trans retinoic acid (10 μM), extracts or single

compounds. At the end of the incubation, luciferase activity was examined using a luminometer as manufacturer's instructions. The luciferase activity was normalized by β-gal activity for transfection efficiency.

3. Preparation of soybean extracts and isolation of active compounds

Dried Soybeans, Glycine max and Rynchosia nulubilis, were collected in Paju, Gyeonggi-do, South Korea. A voucher specimen was deposited at the Amorepacific R&D Center, Yongin, Gyeonggi-do, South Korea, where it is stored at 4°C. Dried Soybeans were ground and extracted with ten volumes of 70% ethanol for 2 days at room temperature. The extracts were obtained by filtering and evaporating the solvent. Each extract was sequentially fractionated with dichloromethane, ethyl acetate, butyl alcohol, and water. The ethyl acetate fractions were applied to silica gel open column chromatography (70-230 mesh, Merck & Co., Whitehouse Station, NJ) and eluted with chloroform and ethyl acetate (10:0.5, v/v) to give five fractions (Fraction I to V). Fraction III and fraction IV was subjected to Rp-18 column chromatography (LiChroprep® RP-18 25-40 μm, Merck & Co.) and eluted with 70% methanol, yielding single compounds, III-A and IV-A, respectively. Data obtained from proton nuclear magnetic resonance (1H-NMR), 13C-NMR, and fast atom bombardment mass spectrometry analysis, identified the compounds

III-A and IV-A as genistin and daidzin, respectively (Fedoreyev et al., 2008).

4. Fluorescence resonance energy transfer (FRET)assay

To analyze binding affinity of isoflavonoids to RAR, FRET assays were performed using LanthaScreen TR-FRET RARα and RARɣ coactivator assays (Invitrogen, Madison, WI). Briefly, various concentrations of ATRA and isoflavonoids were incubated in a solution containing the ligand binding domain of RAR and fluorescein-tagged co-activator peptide. After incubation for 30 min at room temperature, the mixtures were analyzed on a microplate reader with background emission at 490 nm, and binding signal at 520 nm after excitation at 340 nm. The ratio of emission signals at 520 nm and 490 nm were plotted against the log of the compound concentrations and fitted to a sigmoidal dose-response curve using Graphpad prism 5.0 (GraphPad Software, San Diego, CA).

5. RNA preparation and quantitative real time PCR

Total RNA was isolated from cells using EASY-BLUE Total RNA Extraction Kit (iNtRON Biotechnology, Korea) according to manufacturer's instructions. cDNA was synthesized from RNA in a reaction mixture containing M-MLV reverse transcriptase (Invitrogen,

USA). Real time PCR was performed using SYBR Green PCR kit (Applied Bioscience, Warrington, UK) with specific primers. Primers used for qRT-PCR were: forward, 5'-CTC CAT GCC GCC TCT CAT-3' and reverse, 5'-CGG CTG TCC GCT CAG AGT-3' for RARα, forward, 5'-TGT GCG AAA TGA CCG GAA C-3' and reverse 5'-CTA ACT GAG GGC TCA GCT CAT AGC-3' for RARɣ, forward, 5'-CCT GGA GAC CTG AGA ACC AAT C-3' and reverse, 5'-GAT TTC GAC TCT CCA CGC ATC-3' for MMP-9, forward, 5′-CGT GGG CCG CCC TAG GCA CCA-3′ and reverse 5′-TTG GCT TAG GGT TCA GGG GGG-3' for β-actin.

6. Western blot

Cells were washed with PBS and the washed cells were lysed in a lysis buffer containing 150mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 1% NP-40, and protease inhibitor for 30min on ice. Whole-cell lysates were obtained by centrifugation at 14,000 x g for 10 min. The protein concentration was quantifed by BCA assay (Pierce). The 20 mg of the proteins were separated by 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in phosphate-buffer saline containing 0.1% Tween-20 (PBS-T) for 1h and incubated with anti-RARα (sc-551), anti-RARɣ (sc-550) or anti-α-tubulin (Millipore, Billerica, MA) for overnight at 4℃. Then membranes were washed

three times with PBS-T and incubated with secondary antibodies for 1h at room temperature and washed three times with PBS-T again. Bands were visualized by using enhanced chemiluminescence assay kit (Amersham Biosciences).

7. ChIP assay

ChIP assays were performed as described previously (Lee et al., 2012). Nuclear lysates were sonicated, and the lysates were immunoprecipitated using anti-RARα (sc-551), anti-RARɣ (sc-550), anti-p300 (sc-585) and normal IgG antibodies for overnight at 4℃. DNA was prepared by phenol-chloroform extraction and amplified by PCR using specific primer corresponding to the RAR binding site on the RARɣ2 promoter : forward, TCG AAG AAG GAG CAT CGA and reverse, CCG TGA ACT CTC TGG GAA.

8. Zymography for MMP-9

HaCaT cells were seeded in a 100 mm dishes at 1.5×10^6 cells per dish and cultured for overnight. Cells were pretreated with daidzein for 24 h and continued for incubation with TNFα (10ng/ml) for 24 h. After treatment, culture media were concentrated about 10-fold using a 30 KDa cut-off Amicon filter (Millipore, Billerica, MA). Concentrated media were applied for electrophoresis on a 10%

SDS-PAGE containing 0.2% (v/v) gelatin. After incubation at 37℃ for 36 h, the gel was stained with Coomassie blue (Invitrogen) and the MMP-9 band intensity was measured using LAS-1000 plus (Fuji, Tokyo, Japan).

9. Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was determined by unpaired student's t-test. Differences were considered statistically significant when p value was < 0.05.

Ⅳ. RESULTS

1. Soybean extracts and EA fractions of extracts increased RAR transcriptional activity.

Extracts of soybeans, *Glycine max* and *Rhynchosia nulubilis,* increased transcriptional activity of RARα and RARɣ when measured by reporter gene analysis. The activity of RAR activity at 50 μg/ml was about 70% to 90% of the activity induced by 10 μM ATRA (Figure 3A). To identify active components in the soybean extracts, the extracts were further serially fractionated in four parts using water, butyl alcohol, ethyl acetate (EA), and dichloromethane (DCM). Among the fractions, the EA fractions in both soybean extracts induced transcriptional activity of RARα and RARɣ (Figure 3B and Figure 3C). The EA fraction was further fractionated into five fractions by silica gel open column chromatography and fraction III and fraction IV was subjected to Rp-18 column chromatography (LiChroprep® RP-18 25-40 μm, Merck & Co.). Finally single compounds, III-A and IV-A, were obtained and identified as genistin and daidzin, respectively, using ^{1}H-NMR, 13C-NMR, and fast atom bombardment mass spectrometry analysis (Figure 4).

2. Soy isoflavones induced transcriptional activity of RARα and RARγ.

In plants, isoflavonoids are predominantly present as β-glucoside conjugates which have weaker biological activities than aglycones because the conjugates are more polar and water-soluble. Daidzin and genistin are hydrolyzed by β-glucosidases releasing aglycones, daidzein and genistein, respectively, which are easily absorbed and exert their physiological functions (Pilšáková L et al., 2010). We examined the RAR transactivation function of dadzin and genistin and their aglycones. Daidzin and daidzein induced the reporter activity in a dose-dependent manner; the activation of RAR was observed at as low as 0.5 μM and reached maximum at 100 μM. Similar activities were obtained for both RARα and RARγ. Genistin and genistein also activated transcactivation function of RARα and RARγ, but their effects were weaker than that of daidzin and daidzein (Figures 5B).

3. Daidzein is an agonistic ligand of RARα and RARγ

Isoflavonoids could modulate RAR activity directly by binding to ligand binding pocket or indirectly via the induction of endogenous ligands. To examine the direct binding of isoflavones to RAR, we performed a cell-free time-resolved fluorescence resonance energy transfer (TR-FRET) assay. TR-FRET assay provides an indication

of ligand-dependent coactivator recruitment on the RAR ligand binding domain, as measured by an increase in TR-FRET signal. Among the tested isoflavones, only daidzein increased the TR-FRET signals for both RARα and RARɣ. The EC_{50} of binding for RARα and RARɣ were 28 μM and 40 μM, respectively. These results showed that daidzein is an agonistic ligand of RAR which exhibit a low binding affinity compared with that of ATRA (Figure 6A). The docking score of daidzein in the RAR ligand binding pocket also revealed that daidzein is a relatively weak ligand compared to ATRA (Figure 6B).

4. Daidzein enhances mRNA level of RARα and RARɣ in HaCaT cells

To further confirm the RAR agonistic characteristics of daidzein, we examined whether daidzein increased mRNA level of RARα and RAR ɣ mRNA. The expression of RARs are up-regulated by ATRA treatment since RARs themselves are RA target genes which have RAREs in their promoter regions (Leroy et al., 1991, Lehmann et al., 1992). As shown in Figure 7A, daidzein increased RARα and RARɣ mRNA level in a time-dependent manner in HaCaT cells. Daidzein treatment increased the mRNA levels of RARα and RARɣ about 2.5- and 2.0-fold, respectively, in HaCaT cells (Figure 7A). ChIP assay showed that RARα and RARɣ were recruited on the RARE in RARɣ2

promoter, when cells were treated with daidzein (Figure 7B). A coactivator of p300 was also recruited on the promoter upon daidzein treatment, further supporting the agonistic function of the isoflavone. Unlike mRNA level, protein level of RAR decreases after ligand treatment by ligand binding and subsequent ubiquitination/proteasomal degradation (Zhu et al., 1999, Tanaka et al., 2001). This paradoxical phenomenon could be explained that ligand-induced receptor catabolism removes inactivated RAR and relieves transcriptional repression. In addition, this breakdown of receptors may provide the feedback mechanism allowing sustained target gene activation (Zhu et al., 1999). Treatment of ATRA led to a decrease in RAR protein level in a time-dependent manner. Similarly, daidzein decreased the RAR protein level as early as 3 hours and the decrease was continued up to 48 h (Figure 7C). Together, these results indicate that daidzein functions as a RAR ligand in HaCaT cells.

5. Daidzein suppresses the expression and enzymatic activities of MMP-9.

MMP-9, subclassified as gelatinase B, is a member of the MMP family, which degrades gelatin and type IV collagen, the major constituent of basal membrane (Bahar-Shany et al., 2010). It is well-known that ATRA down-regulates the expression of MMP-9, and thereby enhance elasticity in epidermis in skin (Lateef et al.,

2004, Zeng et al., 2011). We next examined whether daidzein affected the expression of MMP-9 in HaCaT cells. Treatment of TNFα (10 ng/ml) largely induced the mRNA level of MMP-9, whereas co-treatment with daidzein decreased dose-dependently the TNFα -induced MMP-9 (Figure 8A). Consistently, a gelatin zymography demonstrated that activity of MMP-9 that induced by TNFα was decreased by pretreatment of dadzein (Figure 8B).

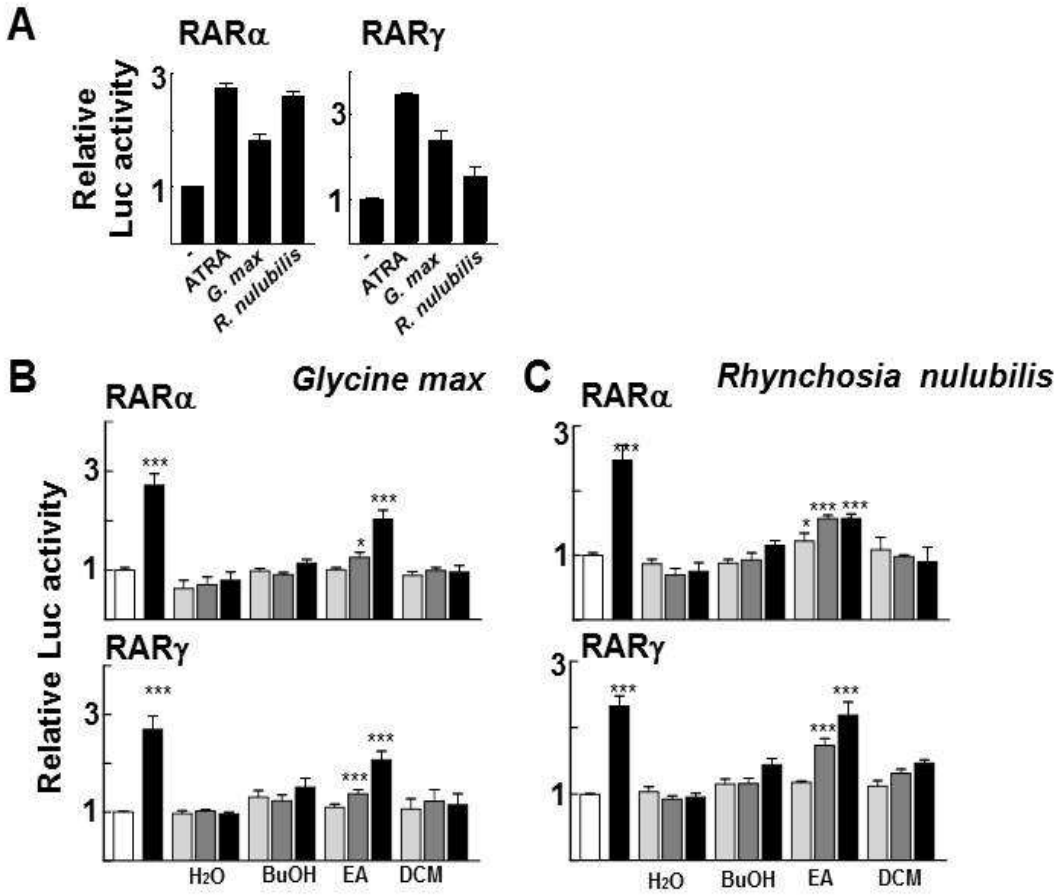


Fig 3. Effects of soybean extracts on transcriptional function of RARa and RARg.

(A) CV-1 cells were transfected with 100 ng ERE-Luc and 50 ng of ER-RARα or ER-RARɣ. Transfected cells were treated with the 70% ethanol extract of *Glycine max* or *Rhynchosia nulubilis* at concentration of 50 μg/ml for 24 h. The β-Gal activity was used to normalize luciferase activity. (B), (C) After transfection, CV-1 cells were treated with each fraction of water, buthanol (BuOH), ethyl acetate (EA), or dichloromethane (DCM) for 24 h. 10 μM ATRA was employed as control. Data represent the means ± SEM (n=4). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with vehicle control.

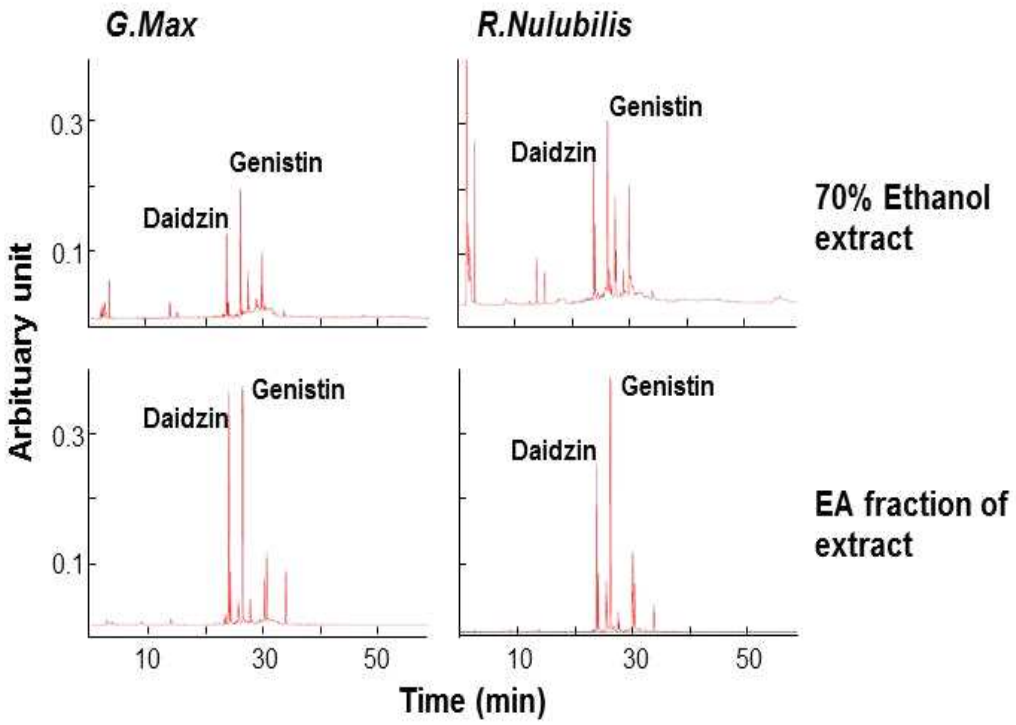


Fig 4. HPLC profilings of the soybean extracts and the EA fractions.

Each extract and fraction was loaded on a column (Mightysil RP-18 GP, 250 X 4.6 ID, 5 μm) and absorbance was measured at 254 nm. A gradient elution was carried out with water/0.1% acetic acid (solvent A) and acetonitrile/0.1% acetic acid (solvent B) at a constant flow rate of 1.0 ml/min. A linear gradient elution program was as follows: 0 - 15 min, 5 - 10% B; 15 - 30 min, 10 - 40% B; 30 - 45 min, 40 - 95% B;45 - 52 min, 95 - 95% B; 52 - 57 min, 95 - 5% B; 57 - 60 min, and back to 5% B.

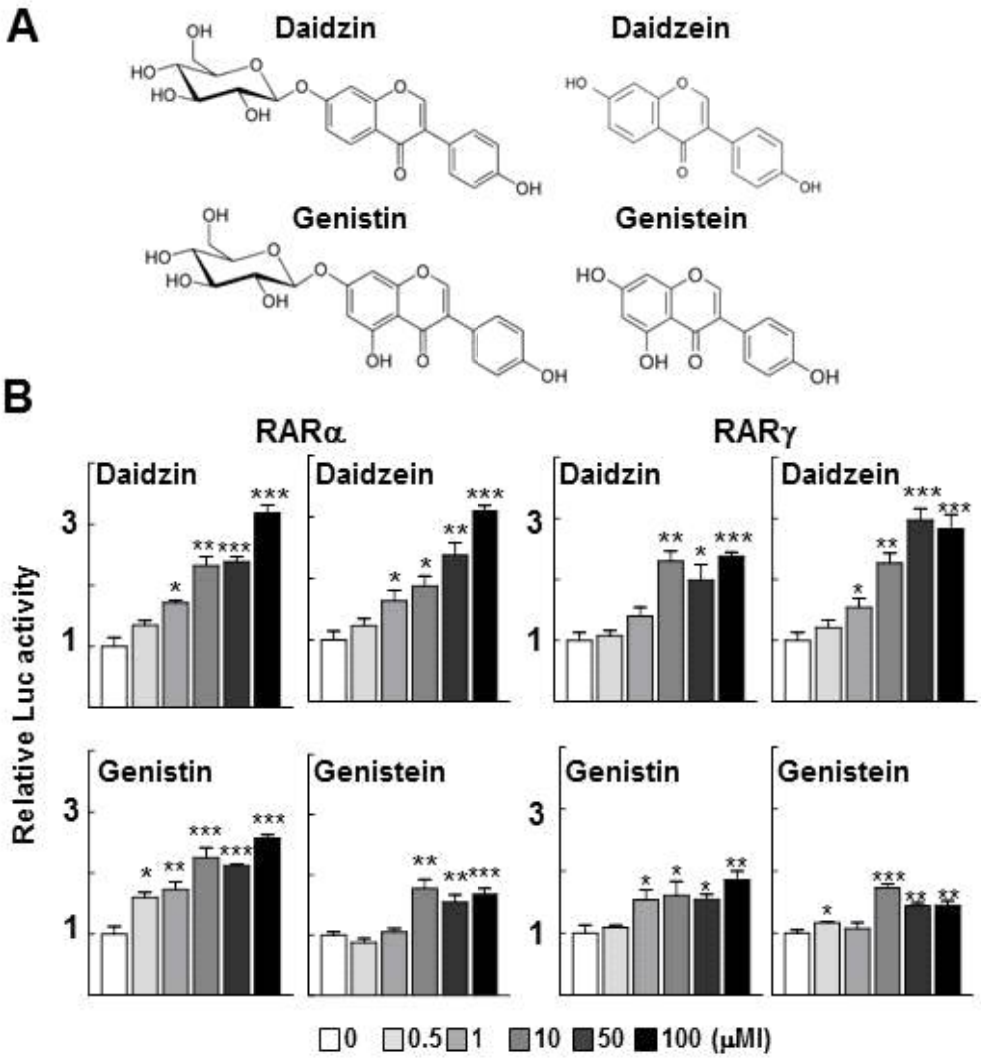
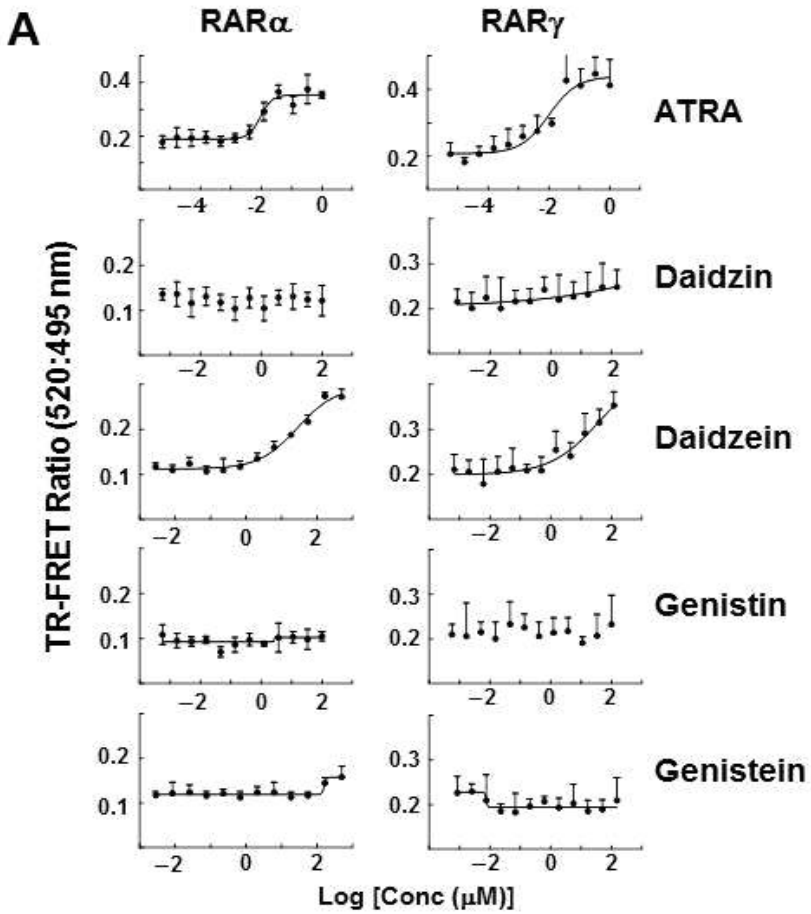


Fig 5. Transcriptional activation of RARα and RARγ by daidzin and genistin and their aglycones.

(A) Chemical structure of daidzin, daidzein, genistin and genistein. (B) CV-1 cells were transfected as described in materials and method. Transfected cells were treated with isoflavonoids for 24 h. The β-Gal activity was used to normalize luciferase activity. Data represent the means ± SEM (n=3). *P<0.05, **P<0.01, and ***P<0.001 compared with vehicle control.


A
RARα
RARγ
ATRA
Daidzin
Daidzein
Genistin
Genistein
TR-FRET Ratio (520:495 nm)
Log [Conc (μM)]

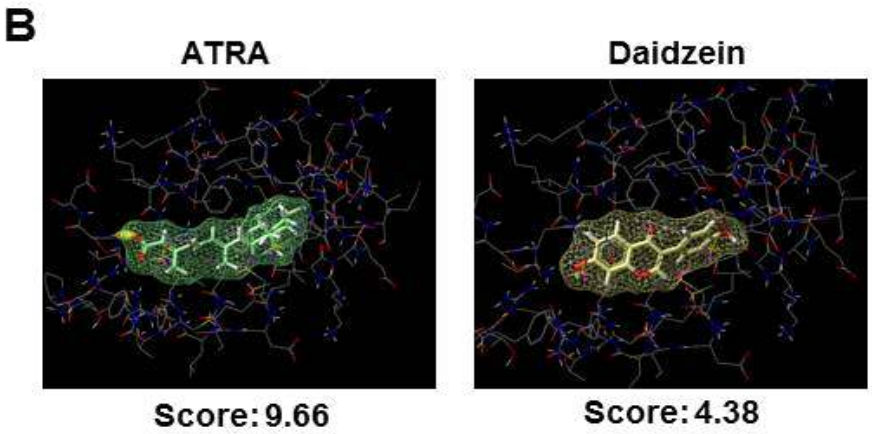

B
ATRA
Daidzein
Score: 9.66
Score: 4.38

Fig 6. Daidzein directly binds to RARα and RARɣ

(A) TR-FRET assay was performed using Lanthascreen RARα or RARɣ coactivator assay kit. Y-axis represents ratio of fluorescence intensity of 520 nm (signal) and 495 nm (background). X-axis represents log scale of isoflavonoid doses. ATRA was used as positive control. Data represents mean ± SD (n=4). (B) Docking model of ATRA and daidzein in the RARα LBD.

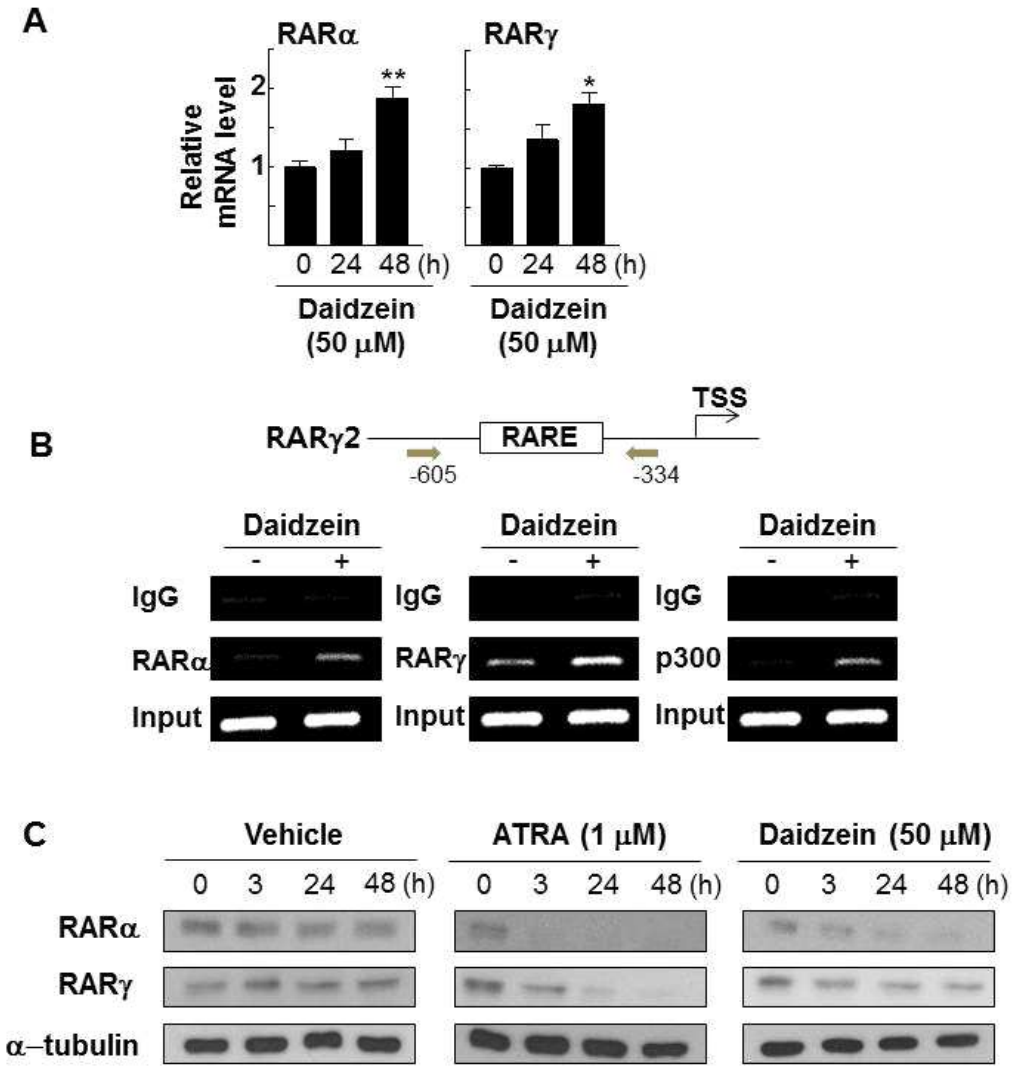

A
RARα
RARγ
Relative mRNA level
2
1
**
*
0 24 48 (h)
0 24 48 (h)
Daidzein (50 μM)
Daidzein (50 μM)
B
RARγ2
RARE
TSS
-605
-334
Daidzein
- +
IgG
RARα
Input
IgG
RARγ
Input
IgG
p300
Input
C
Vehicle
ATRA (1 μM)
Daidzein (50 μM)
0 3 24 48 (h)
RARα
RARγ
α–tubulin

Fig 7. Daidzein increases RAR mRNA level.

(A) HaCaT cells were treated with 50 μM daidzein for indicated time period measured by qRT-PCR. Data represent the means ± SEM (n=3). *P<0.05 compared with vehicle control. (B) Schematic representation of primers used for ChIP assay (upper). HaCaT cells were treated with vehicle or 50 μM daidzein for 48 h. ChIP assay was performed with specific antibodies against RARα, RARγ, and p300 (lower). (C) HaCaT cells were treated with vehicle, 1 μM ATRA or 50 μM daidzein for the indicated time period. Whole cell lysates were obtained and the protein expression of RARα and RARγ were analyzed by Western blotting. α-tubulin was measured as loading control.

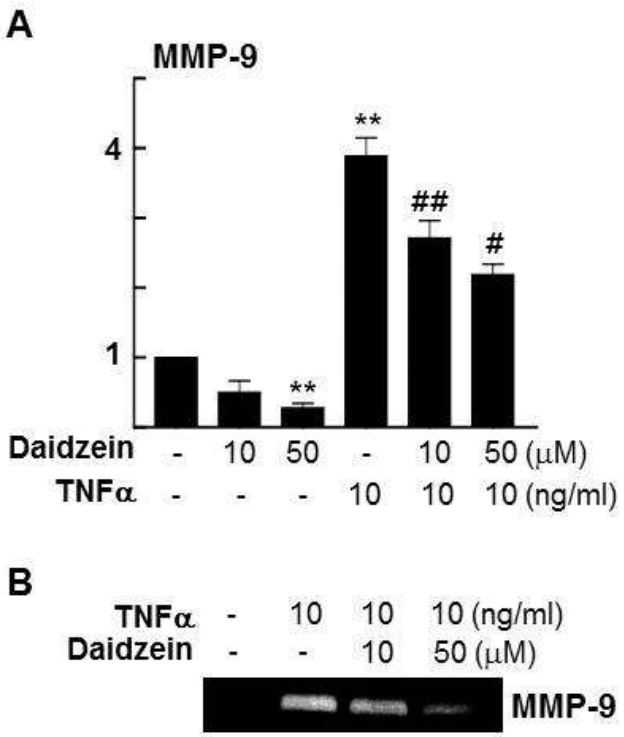


Fig 8. Daidzein decreases mRNA level as well as enzymatic activity of MMP-9.

(A) HaCaT cells were treated with 10 or 50 μM daidzein for 36 h and then exposed to TNFα (10 ng/ml) for 12 h. At the end of incubation, total RNA was isolated and mRNA level of MMP-9 was measured by qRT-PCR. Data represent the means ± SEM (n=3). *P < 0.05, and **P < 0.005 compared with vehicle control. #P < 0.05 compared with TNFα alone. (B) HaCaT cells were treated with 10 or 50 μM daidzein for 24 h and then exposed to TNFα (10 ng/ml) for 24 h. Gelatinolytic activity of MMP-9 in media was analyzed by zymography.

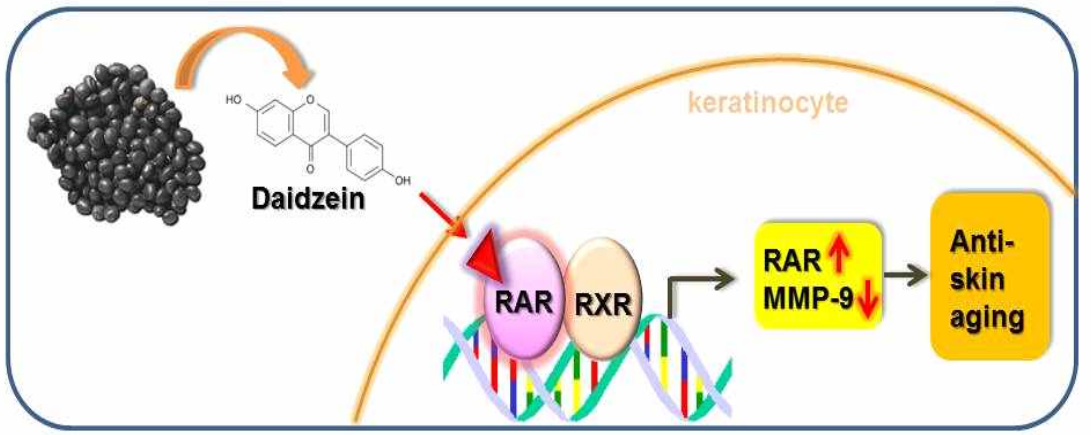


Fig 9. Schematic illustrations of functions of daidzein in the keratinocyte.

Daidzein, one of soy isoflavones, acts as an agonistic ligand of retinoic acid receptor and regulates retinoic acid related genes in the keratinocyte, suggesting the novel mechanism of soy isoflavones actions in the skin.

Ⅴ. DISCUSSION

Retinoids are widely used as therapeutic ingredients for diverse skin diseases including skin aging, acne, psoriasis (Thacher et al., 2000). Although its efficacy, unwanted side effects such as skin irritation and photosensitivity limit their applications to clinical usage (Ferguson and Johnson., 1989, Griffiths et al., 1995). Many efforts have been made to find retinoids derivatives or their alternatives with less side effects, and to develop additives to reduce retinoid dermatitis. For instance, soy bean extracts and soy isoflavones are known to reduce retinoid-induced hyperplasia when treated with retinoic acid (Varani et al., 2004). Here, we demonstrated that one of soy isoflavones, daidzein is a new natural RAR ligand and regulates retinoic acid related genes in the keratinocyte.

Two types of soybean extracts, *Glycine max* and *Rhynchosia nulubilis*, and their EA fractions activated the transcriptional activities of both RARα and RARɣ (Figure 3). These data are in good accordance with a recent study showing that soybean extracts modulated retinoic acid related genes in skin (Park et al., 2012). Daidzin and genistin were major single compounds in EA fractions and ethanol extracts, analyzed by HPLC (Figure 4).

To identify which isoflavones regulate RAR transcriptional activity,

we examined the both of glycoside-conjugates and aglycone forms of isoflavones. Our results showed that tested four isoflavones increased RAR activity but their ability of activation were differ. Daidzin and daidzein were more effective activators of RARs than genistin and genistein (Figure 5B). The TR-FRET assay revealed that only the daidzein binds directly to ligand binding domains of both RARα and RARɣ and induces a conformational change like all-trans retinoic acid (ATRA) that recruits co-activators (Figure 6A). Daidzein also regulates RA target genes in the keratinocyte (Figure 7). The binding affinity (EC_{50}) is much smaller than that of ATRA and the binding score of daidzein in docking study is also lower than ATRA (Figure 6B). These results showed that daidein is a much weaker ligand of RAR compared to ATRA. Daidzein binds RARα stronger than RARɣ, which the values of EC_{50} were 28 μM and 40 μM, respectively. As shown in Figure 5B, daidzin also increased RAR transcriptional activity similar to daidzein. We speculate that daidzin may be hydrolyzed by cytoplasmic β-glucosidases releasing daidzein and exert RAR activation effect (Day et al., 1998). Previous studies reported that β-glucosidases are also expressed and have functions in mammalian epidermis (Ohkawara et al., 1972, Wertz and downing., 1989, Chang et al., 1993). Although soy extracts contain soy isoflavones mainly as glucoside-conjugates forms, applications of soy extracts to skin could exert RAR activation effect as these β-glucosidases would convert daidzin to daidzein.

Genistin and genistein do not bind to RARα and RARɣ LBD. In case of estrogen receptor, both genistein and daidzein bind to ERα and ER β, but the binding affinity of genistein is much stronger than daidzein. They both preferentially bind to ERα than ERβ (Kuiper et al. 1997). Together with our observations, although daidzein and genistein have similar chemical structures, they might have distinct characteristics as nuclear receptor ligands. These different actions of daidzein and genistein could lead to different applications of two isoflavones for the purpose of treatment. When retinoic acid receptor activations are needed, such as treatment of acne vulgaris, daidzein should be treated instead of genistein.

Other natural RAR activators that have distinct structures from retinoic acid were found previously. Lycopene and its metabolite apo-10'-lycopenic acid have been reported to induce RAR transcriptional activity in vivo and in vitro (Aydemir et al., 2012, Gouranton et al., 2011). Although they transactivate RARs and modulate RAR target gene transcription, unlike daidzein in our study, direct interaction with the RARs and lycopene and its metabolites have not been identified. Due to the large shape of lycopene and apo-10-lycopenoic acid, further smaller metabolites which have not been found, are expected to bind and regulate RARs. Recently found another natural RAR ligands, lufariellolide, have similar structures like ATRA, possessing a hydrophobic chain and a trimethylcyclohexane group. It directly binds to RAR LBD and regulates RAR target gene

transcription (Wang et al., 2012). Lufariellolide is more potent than daidzein as their effective concentration is 1 μM, compared to 50 μM of daidzein in our experiments.

Daidzein treatment attenuated MMP-9 mRNA level and gelatinolytic activity induced by TNFα (Figure 8). MMP-9, an inducible gelatinase, is expressed and secreted by keratinocytes when they are in stress. Persistent up-regulation of MMP-9 disturbs the balance between tissue degradation and formation, and leads to loss of elasticity in epidermis, increase migration of immune cells and activate chemokines and cytokines (Ladwig et al., 2004, Van lint and libert., 2007, Purwar et al., 2008). Molecular mechanism of daidzein effect in MMP-9 expression is not clear in our study. However, it is known that retinoids reduce MMP-9 expression via reducing EGFR and NF-kB signaling and treatment of retinoids to the diabetic patient skins which have higher risk of chronic skin ulceration decreases the elevated MMP-9 expression (Dutta et al., 2010, Lateef et al., 2004, Zeng et al., 2011). Further examinations using knockdown of RARα/ɣ would help to clarify the role of RARs in daidzein effects of MMP-9 attenuation. Up regulation of MMP-9 is also related to diverse diseases including cancer metastasis, rheumatoid arthritis, and asthma (Muroski et al., 2008). Daidzein effect of suppression of MMP-9 could be employed not only skin aging but also other diseases such as rheumatoid arthritis.

Taken together, the identification of daidzein as a natural novel agonist of RAR and its effect in suppression of MMP-9 may provide new drug design strategies to overcome retinoids side effects in skin and diverse other diseases.

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국 문 초 록

레티노이드는 피부에서 각질형성세포의 증식과 분화, 피지 분비 조절, 면역 및 항염증 작용을 하여 피부 노화, 여드름과 아토피 등의 치료제로서 사용되고 있다. 이러한 레티노이드의 생체 내에서의 작용은 리간드 의존적인 전사 인자인 레티노이드 수용체 (Retinoic acid receptor, RAR)에 의해 매개된다. 따라서 레티노이드 수용체를 활성화시킬 수 있는 리간드는 다양한 피부질환에서 레티노이드와 같은 역할을 할 것이라고 예상할 수 있다. 본 연구에서는 Soy isoflavone인 daidzein이 레티노이드 수용체의 리간드로서 작용한다는 것을 규명하였다. 먼저, 서리태 (Glycine max)와 서목태 (Rhynchosia nulubilis)의 70% 에탄올 추출물 50 μg/ml에서 RARα와 RARɣ가 활성화되는 것을 리포터 유전자 활성 실험을 통해 확인하였다. 각 콩 추출물을 에칠아세테이트 (Ethyl acetate, EA), 물, 부탄올과 다이클로로메테인 (Dichloromethane, DCM)으로 분획하여 확인해 본 결과 EA 분획물에서 농도-의존적으로 레티노이드 수용체가 활성화되었다. Daidzin과 genistin이 콩 추출물과 EA 분획물의 주요 성분이라는 것을 HPLC 분석을 통해 밝혔다. Daidzin과 아글리콘인 daidzein은 농도 의존적으로 레티노이드 수용체를 활성화시켰고, genistin과 아글리콘인 genistein의 경우에서도 레티노이드 수용체를 활성화시키나 daidzin과 daidzein에 비하여 그 정도가 약하다는 것을 확인하였다. Time-resolved fluorescence resonance energy transfer (TR-FRET) 분석을 시행해 본 결과 daidzein이 RARα와 RARɣ의 리간드 결합 부위에 직접적으로 결합하며 EC_{50}값은 각각 28 μM와 40 μM이었다. 피부 세포주인 HaCaT을 이용한 실험에서 daidzein 50 μM을 처리하였을 때 RAR의 타겟 유전자인 RARα, RARɣ의 mRNA 레벨이 증가하였고, 단백질의

발현은 시간이 지남에 따라 감소하는 패턴을 확인하였다. 콜라겐을 분해하여 피부 노화를 촉진시키는 matrix metallopeptidase (MMP) 중 하나인 MMP-9의 경우 TNFα처리에 의해서 발현이 증가되지만 daidzein 처리를 하였을 때에는 발현이 감소하였다.

이와 같은 연구 결과는 soy isoflavone인 daidzein이 레티노이드 수용체의 리간드로서 작용하며 이는 콩 추출물과 daidzein의 항 피부 노화 효능의 기전임을 제시한다.

주요어 : 피부, 레티노이드 수용체, 리간드, daidzein

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