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Effect of Genistein on the Aryl Hydrocarbon Receptor and Cytochrome P450 1A1 in MCF-7 Human Breast Carcinoma Cells

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인체유방암 세포주 MCF-7 세포에서 genistein의 Aryl Hydrocarbon Receptor와 Cytochrome P450 1A1에 대한 영향

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요 약

화학적 예방효과가 있는 식물성 에스트로젠은 다양한 활성을 나타내며 여러 세포 수용체와 상호작용한다. Genistein은 isoflavone의 주요물질 중의 하나로 콩류에 존재하며 대표적인 식물성 에스트로젠이다. 본 논문에서는 유방암 세포주인 MCF-7에서 aryl hydrocarbon receptor (AhR)에 의해 매개되는 발암물질 활성화 경로에 대한 genistein의 영향을 살펴보았다. 세포에 genistein을 처리할 경우 cytochrome P450 1A1 (CYP1A1) 약물대사효소의 특이적인 효소반응인 7-ethoxyresorufin *O*-deethylase (EROD) 활성도와 CYP1A1의 유전자 발현이 genistein의 농도 의존적으로 증가하였다. Genistein과 발암물질인 방향족탄화수소 7, 12-dimethylbenz[a]anthracene (DMBA)를 동시 처리하였을 경우 DMBA에 의해 유도되어 증가된 EROD 활성도와 CYP1A1의 유전자 발현이 genistein에 의해 감소하였다. 랫트의 간에서 분리한 세포질을 이용하여 genistein과 AhR의 대표적인 ligand인 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin과 경쟁적 결합에 대한 영향을 조사한 결과 genistein이 AhR에 경쟁적으로 결합함을 알 수 있었다. 이러한 결과들은 genistein이 천연 AhR ligand임을 암시한다. 따라서, 식물성 에스트로젠인 genistein은 AhR 경로의 길항제/항진제로 작용할 수 있을 것으로 사료된다.

Key words : genistein, CYP1A1, aryl hydrocarbon receptor, 7, 12-dimethylbenz[a]anthracene

INTRODUCTION

The aryl hydrocarbons receptor (AhR) is a ubiquitous cytosolic protein that binds environmental contaminants such as polycyclic aromatic hydrocarbons (PAH) i.e. 7, 12-dimethylbenz[a]anthracene (DMBA) and benzo(a)pyrene, and halogenated derivatives such as 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). Upon binding ligand, the AhR translocates to the nucleus where it binds another

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protein, the aryl hydrocarbon nuclear translocase (ARNT). This heterodimer acts as a transcription factor of the basic helix-loop-helix family of DNA binding proteins. It binds to enhancer sequences, which are known as dioxin-response elements (DREs) flaking the 5′-promoter region of several genes (Hankinson, 1995). DREs are located upstream of the CYP1A1 transcription start site (Brotons *et al.*, 1995). The binding to these enhancer sequences causes a change in the chromatin structure, which facilitates the binding of various transcription factors to the CYP1A1 promoter (Olea *et al.*, 1996). The most extensively studied cellular response to PAH is the transcriptional induction of the gene CYP1A. This gene encodes the enzyme cytochrome P4501A, which catalyzes the oxidative catabolism of PAH. The transcriptional induction of the CYP1A gene is the most comprehensively studied cellular response to PAH. This reaction generates genotoxic metabolites that can enter the nucleus and bind to specific DNA residues, leading to mutagenesis (Dipple, 1994). The level of P450 gene expression of these enzymes is influenced by the number of endogenous regulatory factors, such as hormones, as well as by their xenobiotic substrates including natural and synthetic chemicals (Hankinson, 1995). 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent environmental contaminant, which has been used as a model compound for examining the mechanisms of Ah action. Also, 7, 12-dimethylbenz[a]anthracene (DMBA), which is a model compound that induces mammary tumorgenesis in rodents (Brotons *et al.*, 1995), is a member of one such class of carcinogens, PAH. The inhibition of the CYP1 enzymes appears to be beneficial in preventing the formation of DMBA-DNA adducts both *in vivo* and *in vitro* (MacDonald *et al.*, 2001). Epidemiological studies have shown that a polymorphism of increased CYP1A1 expression is a risk factor for breast cancer (Taioli, 1999).

Flavonoids are part of a family of naturally occurring polyphenolic compounds and represent one of the most prevalent classes of compounds in vegetables, nuts, fruits and beverages such as coffee, tea, and red wine (Hollman and Katan, 1997) as well as medical herbs. Genistein is a phytoestrogen that occurs naturally in the diet especially in soybeans and soy-based foods. Genistein and related phytoestrogens are of interest as chemopreventive agents for a variety of diseases and cancers based on epidemiologic evidence of reduced cancer rates in populations with a high intake of soy (Cos *et al.*, 2003). Experimental findings suggest that phytoestrogens play a significant inhibitory role during the initiation and promotional phases of cancer development (Cho *et al.*, 2004). Genistein is a methyl derivative of genistein, and has been reported to protect against mammary carcinogenesis in rats, and mammary tumor virus-induced spontaneous breast cancer in mice (Hollman and Katan, 1997). In the present study, we have used the human mammary epithelial carcinoma MCF-7 cell line to examine the interaction between genistein and the AhR pathway. We report, for the first time, that genistein also to induce accumulation of CYP1A1 mRNA and induce CYP1A1 enzymatic activity by activation of the AhR. Genistein is able to compete with TCDD for binding to isolated AhR, and partially inhibits the response of the AhR to DMBA. These results indicate that genistein is a ligand of the AhR and possibly a substrate of CYP1A1, and is only the second natural dietary factor to be so identified. In the present study, we investigated the action of genistein with the carcinogen activation pathway that is mediated by the AhR in MCF-7 cells.

MATERIALS AND METHODS

1. Materials

All chemicals and cell culture materials were obtained from the following sources: genistein (>99% pure: Sigma); [^{3}H]TCDD was purchased from ChemSyn.; 7-ethoxyresorufin and resorufin (Pierce Chemical Co.); DMBA (Chemsyn Science Lab.); RPMI 1640, fetal bovine serum, penicillin-streptomycin solution, and trypsin (Life Technolo-

gies, Inc); Liquid scintillation cocktail (Fisher); charcoal-dextran (Sigma).

2. Cell culture and treatment

The MCF-7 cells were grown in RPMI 1640 supplemented with 2 mM of glutamine and 10% fetal bovine serum. The cells were subcultured weekly using 0.25% trypsin/0.05% EDTA. Unless indicated otherwise, all the experiments were carried out using confluent cells in a growth medium. Both genistein and DMBA were dissolved in dimethylsulfoxide (DMSO). Stock solutions of these chemicals were added directly to the culture media and incubated with genistein and DMBA. The control cells were treated with the DMSO only, and the final concentration of the solvent was always $< 0.2\%$.

3. 7-Ethoxyresorufin-*O*-deethylase assay

The MCF-7 cells in 48-well plates were treated with genistein at the concentrations indicated in the figures with or without 1 μM of DMBA in a growth medium for 18 hr. After incubation, the medium was removed and the wells were washed twice with fresh medium. The 7-Ethoxyresorufin-*O*-deethylase (EROD) activity was determined in the intact cells grown in 48-well plates, as described elsewhere (Juchau, 1990). The fluorescence was measured every 10 min for 60 min using a FL600 ELISA reader (BIO-TEK), with excitation at 530 nm and emission at 590 nm. A standard curve was constructed using resorufin.

4. RNA preparation and CYP1A1 mRNA analysis by RT-PCR

The confluent MCF-7 cells were treated with genistein and/or 1 μM of DMBA in growth medium for the times and concentrations indicated in the figures. The cells were washed twice with PBS, and the total RNA was isolated using the method reported by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). cDNA synthesis, semiquantitative RT-PCR for CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and analysis of the results were performed as described in the literature (Jeong, 1997). The cDNA was synthesized from 0.2 μg of the total RNA using an Omniscript RT-PCR kit according to the manufacturer's instructions. A cycle number that fell within the exponential range of both the CYP1A1 (302 bp, 26 cycles) and GAPDH (983 bp, 17 cycles) responses was used.

5. Preparation of cytosol

Male Sprague-Dawley rats (200 ~ 250 g) were exposed to a 12 hr light/dark cycle and provided with food and water. The hepatic cytosol was prepared at 4°C in a HEDGK buffer (25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 80 mM KCl) according to the method reported by Denison *et al.* (1986) and stored at −80°C until use.

6. AhR ligand binding assay

The binding of genistein to AhR in rat liver cytosolic extracts was measured by determining the ability of genistein to compete with [^{3}H]TCDD for specific binding using the hydroxylapatite (HAP) method essentially as described by Gasiewicz and Neal (1982). In a competition assay, 500 μL of cytosolic extract (3 mg of protein/mL) was mixed with 10 nM [^{3}H]TCDD (22.2 Ci/mmol), and genistein or solvent (DMSO) alone. Samples were incubated with gentle rotation at 4°C for 2 hr. The unbound ^{3}H-labelled compounds were removed by adding 50 μL of a charcoal suspension, followed by incubation at 4°C for 1 hr. After incubation, 200 μL of the sample was placed in a fresh tube containing 250 μL of HAP suspension for the determination of the amount of bound [^{3}H]TCDD. The samples were incubated on ice for 30 min with gentle shaking every 10 min. At the end of this time, 1 mL of ice-cold HEDGK containing 0.5% (v/v) Tween 80 was added to each sample. Liquid scintillation cocktail solution was added to each vial and the radioactivity was quantified by liquid scintillation counting.

7. Statistical analysis

All experiments were repeated at least three times to ensure reproducibility. The results are reported as a mean±S.D. ANOVA was used to evaluate the differences between multiple groups. A Dunnet's *t*-test was used to compare the means of two specific groups if there was a significance difference observed. A P value <0.01 was considered significant.

RESULTS

1. Effects of genistein on CYP1A1 activity

The CYP1A1 gene activity in MCF-7 cells treated with genistein in the presence or absence of DMBA was measured using an EROD activity assay. When the MCF-7 cells were treated with 1 μM of DMBA for 18 hr, there was an increase in the CYP1A1 enzyme activity, as measured by EROD assay. Genistein alone significantly induced the EROD activity in a dose dependant manner (Fig. 1A). Genistein significantly decreased the DMBA-induced EROD activity in a dose dependent manner (Fig. 1B). The genistein-mediated suppression of EROD induction was not the result of a genistein cytotoxic effect (data not shown).

2. Effects of genistein on CYP1A1 mRNA

The CYP1A1 mRNA in MCF-7 cells treated with DMBA in the presence or absence of genistein was measured using RT-PCR. Surprisingly, the genistein treatment alone caused an approximately 3.5-fold increase in the CYP1A1 mRNA level. Treatment with genistein resulted in a dose dependent increase in CYP1A1 mRNA accumulation from 10 to 50 μM (Fig. 2A). Treating the cells with 1 μM DMBA for 6 hr caused a 5.5-fold increase in CYP1A1 mRNA accumulation. Genistein inhibited the DMBA-induced CYP1A1 mRNA in a dose dependant manner (Fig. 2B).

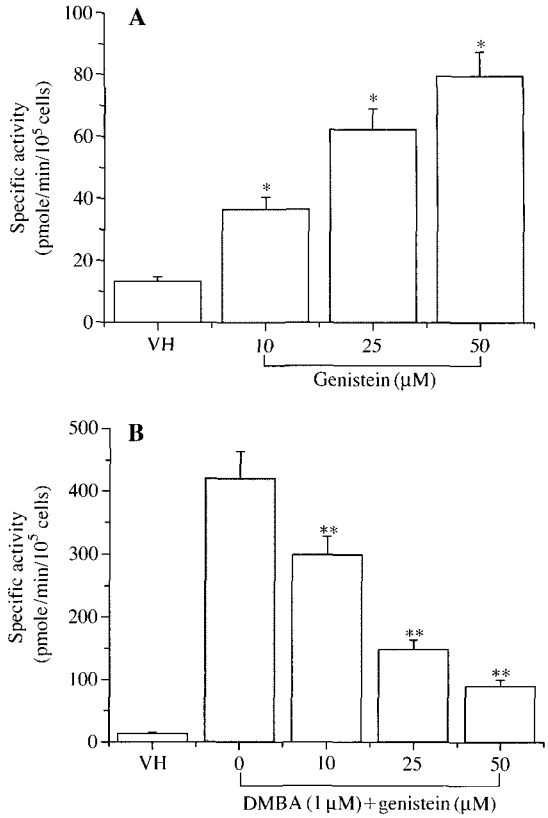

Fig. 1. Effects of genistein on the EROD activity in MCF-7 cells. The cells were treated with DMSO (VH: vehicle), genistein (10~50 μM: A), DMBA (1 μM) plus genistein (10~50 μM: B) for 18 hr, as described in Materials and Methods. The values are reported as a mean±S.D. of triplicate cultures. $*P<0.01$ significantly different from the VH. $**P<0.01$ significantly different from DMBA.

3. Effect of genistein on binding of TCDD to AhR

The EROD and CYP1A1 gene expression data suggests that genistein is a ligand for the AhR. This was further demonstrated by examining the ability of genistein to compete with the prototypical AhR ligand TCDD for binding to the AhR in the cytosolic fraction isolated from rats. As shown in Fig. 3, genistein competitively inhibited specific [^{3}H]TCDD binding to AhR by approximately 74%. These data demonstrate that genistein can compete directly binding to AhR.

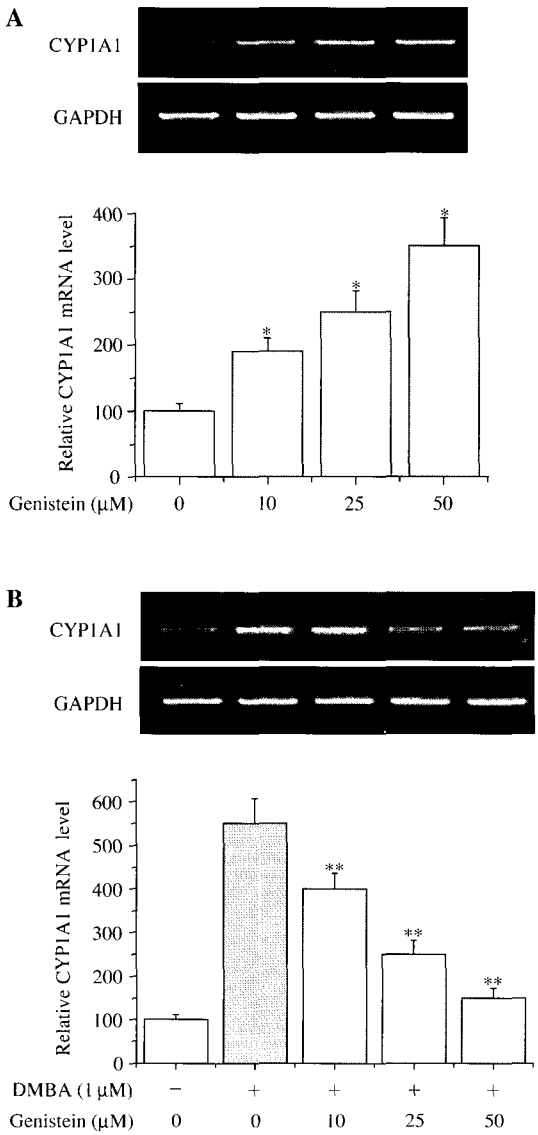

Fig. 2. RT-PCR analysis of CYP1A1 mRNA in MCF-7 cells. The cells were treated with genistein (10~50 μM: A) or DMBA (1 μM) plus genistein (10~50 μM: B) for 6 hr. The total cellular RNA was isolated from the cells. The PCR amplification products were electrophoresed in 2% agarose gel and stained with ethidium bromide. One of three representative experiments is shown. The CYP1A1 levels are shown as the CYP1A1/GAPDH mRNA expression levels and are reported as a mean±S.D. of three representative experiments. The ratio of the RT-PCR products of CYP1A1 to GAPDH was calculated. $*P<0.01$ significantly different from VH. $**P<0.01$ significantly different from DMBA.

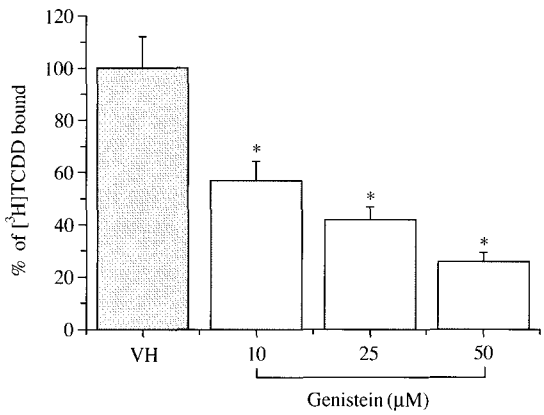

Fig. 3. Genistein competes with [^{3}H]TCDD for binding to cytosolic AhR. Cytosolic extracts (3 mg/mL) containing AhR were incubated for 2 hr at 4°C with 10 nM [^{3}H]TCDD or DMSO (VH: vehicle). The percentage of [^{3}H]TCDD bound for genistein in the competition assay was calculated by dividing the disintegrations per minute of specific [^{3}H]TCDD bound in the genistein containing sample by the disintegrations per minute of specific [^{3}H]TCDD bound in the DMSO containing sample. The values are presented as the mean±S.D. of triplicate cultures. $*P<0.01$ significantly different from DMSO.

DISCUSSION

The isoflavone genistein, a biologically active component of soy foods, is associated with reduced breast cancer risk in women who consume soy-rich diets. Genistein has been reported to influence many biological processes, of which suppression of cell proliferation and stimulation of apoptosis are considered to be the major pathways underlying its inhibition of tumorigenesis. A number of naturally occurring isoflavones have been shown to modulate the CYP450 system, which include the induction of specific CYP isozymes and the activation or inhibition of these enzymes. Some isoflavones alter the CYPs by binding to the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor, acting as either AhR agonists or antagonists. Genistein is of particular interest because it affects other *in vitro* mechanisms that are relevant to chemopre-

vention (Rowell *et al.*, 2005). However, there are no reports of its effect on activate the AhR, induces CYP1A1 mRNA accumulation and CYP1A1 enzymatic activity. Therefore, in this study examined the action of genistein with the carcinogen activation pathway that is mediated by the AhR in MCF-7 breast carcinoma cells. We examined the effect of genistein on the AhR and the major carcinogen-activating enzyme in MCF-7 cells, CYP1A1. The treatment with genistein alone significantly induced the EROD activity (Fig. 1A). DMBA induced CYP1A1 activity in a concentration-dependent manner as measured by the EROD activity. Treatment of the cells with genistein during DMBA exposure resulted in the dose dependent inhibition of CYP1A1 activity (Fig. 1B). Surprisingly, treating the MCF-7 cells with genistein in the absence of DMBA resulted in the increased accumulation of CYP1A1 mRNA. Using RT-PCR, we found that the mammary carcinogen DMBA caused an increase in CYP1A1 mRNA in MCF-7 cells that was partially antagonized by simultaneous treatment of the cells with genistein (Fig. 2A). In addition, mammary carcinogen DMBA caused an increase in the CYP1A1 mRNA in MCF-7 cells that was partially antagonized by simultaneously treating the cells with genistein (Fig. 2B). Genistein, therefore, activates the AhR, induces CYP1A1 mRNA accumulation, and induces CYP1A1 enzymatic activity. These results suggest but do not prove conclusively that genistein is a ligand of the AhR. To determine whether genistein acts similarly or interacts directly with the AhR, a ligand-binding assay was performed. Cytosol isolated from rat was incubated with radiolabeled TCDD in the presence of genistein. Specific binding was separated from nonspecific binding by hydroxyapatite chromatography. As shown in Fig. 3, genistein partially inhibited TCDD binding to the AhR. Thus, genistein appears to be an antagonist of the AhR in the presence of other AhR ligands such as DMBA or TCDD. Inhibition of the AhR-mediated response of CYP1A1 to DMBA and direct inhibition of CYP1A1 activity by genistein would be expected to reduce the metabolic activation of DMBA. Several studies have demonstrated that non-toxic AhR agonists exhibit anti-tumorigenic activity in the DMBA-induced rat mammary tumor model, thereby, representing a group of compounds with potential for clinical treatment of breast cancer (Krishnan *et al.*, 1995; McDougal *et al.*, 1997). Therefore, genistein represents a new class of relatively non-toxic anti-tumorigenic AhR agonists which are of phytochemical origin.

CONCLUSION

Genistein may be involved in the chemopreventive properties, by reducing the formation of carcinogens through inhibition of enzymes, such as CYP1A1, which are known to be involved in carcinogen activation. However, the fact that genistein induces CYP1A1 *via* the AhR and is an inhibitor of CYP1A1 activity suggests that it might be a natural ligand for AhR.

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