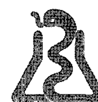
J. Toxicol. Pub. Health
Vol. 23, No. 2, pp. 135-142 (2007)



Official Journal of Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Effects of Formononetin on the Aryl Hydrocarbon Receptor and 7,12-Dimethylbenz[a]anthracene-induced Cytochrome P450 1A1 in MCF-7 Human Breast Carcinoma Cells

Eun Hee Han[1], Tae Cheon Jeong[2] and Hye Gwang Jeong[1]

[1]BK21 Project Team, Department of Pharmacy, College of Pharmacy, and Research Center for Proteineous Materials, Chosun University, Gwangju 501-759
[2]College of Pharmacy, Yeungnam University, Kyungsan, Korea

Received June 6, 2007; Accepted June 27, 2007

Formononetin is an isoflavonoid phytoestrogen found in certain foodstuffs such as soy and red clover. In this study, we examined the action of formononetin with the carcinogen activation pathway mediated through the aryl hydrocarbon receptor (AhR) in MCF-7 breast carcinoma cells. Treating the cells with formononetin alone caused the accumulation of CYP1A1 mRNA as well as elevation in CYP1A1-specific 7-ethoxyresorufin *O*-deethylase (EROD) activity in a dose dependent manner. However, a concomitant treatment with 7,12-dimethylbenz[a]anthracene (DMBA) and formononetin markedly reduced both the DMBA-inducible EROD activity and CYP1A1 mRNA level. Under the same conditions, formononetin inhibited the DMBA-induced AhR transactivation, as shown by reporter gene analysis using a xenobiotic responsive element (XRE). Additionally, formononetin inhibited both DMBA-inducible nuclear localization of the aryl hydrocarbon receptor (AhR) and metabolic activation of DMBA, as measured by the formation of the DMBA-DNA adducts. Furthermore, formononetin competed with the prototypical AhR ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), for binding to the AhR in an isolated rat cytosol. These results suggest that formononetin might be considered as a natural ligand to bind on AhR and consequently produces a potent protective effect against DMBA-induced genotoxicity. Therefore, that's the potential to act as a chemopreventive agent that is related to its effect on AhR pathway as antagonist/agonist.

Key words: Formononetin, 7,12-Dimethylbenz[a]anthracene, CYP1A1, Aryl hydrocarbon receptor.

INTRODUCTION

The aryl hydrocarbons receptor (AhR) is a ubiquitous cytosolic protein that binds environmental contaminants including polycyclic aromatic hydrocarbons (PAH) (7,12-dimethylbenz[a]anthracene (DMBA) and benzo(a)pyrene) and halogenated derivatives such as TCDD. Upon binding ligand, the AhR translocates to the nucleus where it binds another 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 xenobiotic-response elements (XREs) flanking the 5'-promoter region of several genes (Hankinson, 1995). XREs 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 gene expression of CYP enzymes is influenced by the number of endogenous regulatory factors, such as hormones, as well as by

Correspondence to: Hye Gwang Jeong, BK21 Project Team, Department of Pharmacy, College of Pharmacy, and Research Center for Proteineous Materials, Chosun University, Gwangju 501-759, Korea
E-mail: hgjeong@chosun.ac.kr

their xenobiotic substrates including natural and synthetic chemicals (Hankinson, 1995). A model compound of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been used as a potent environmental contaminant for the examining of the mechanisms of Ah action.

One of the most important factors that determine the sensitivity to mammary carcinogenesis is the metabolic stage of the carcinogenic agent. DMBA is a PAH, which is widely used as a model chemical carcinogen in rat mammary tumor model (Chidambaram and Baradarajan, 1996). DMBA is a procarcinogen and requires metabolic conversion to its ultimate carcinogenic diol epoxide metabolites by oxidation, which is carried out through CYP1A1 (Christou *et al.*, 1987; Kleiner *et al.*, 2002). The covalent binding of the diol epoxides of DMBA to DNA is believed to be essential for tumor initiation (Slaga et al., 1979; Chidambaram and Baradarajan, 1996). Therefore, the extent to which the DNA adducts occurs after administering the DMBA depends on the level of the oxidative metabolism of DMBA due to CYP1 (Kleiner *et al.*, 2002). The inhibition of the CYP1 enzyme 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).

Isoflavones are abundant in legumes such as soy, mung bean sprouts, kidney beans, navy beans, red clover, and Japanese arrowroot (Mazur *et al.*, 1998; Horn-Ross *et al.*, 2000). Both protective and adverse health effects have been associated with the consumption of foods and food supplements containing isoflavone derivatives. Dietary consumption of isoflavones has been linked to lower risks for breast cancer (Lamartiniere *et al.*, 1998) and prostate cancer (Adlercreutz *et al.*, 1995). Phytoestrogens from the isoflavone family are found in several plants used for human and animal nutrition (Cos *et al.*, 2004). Experimental findings suggest that phytoestrogens play a significant inhibitory role during the initiation and promotional phases of cancer development (Cho *et al.*, 2004). Phytoestrogen formononetin (4-methoxy-7-hydroxyisoflavone) is the 4-*O*-methyl derivatives of daidzein (4,7-dihydroxyisoflavone), and are the predominant isoflavones found in alfalfa (Horn-Ross *et al.*, 2000), red clover, and chick peas (Mazur *et al.*, 1998). However, the influence of formononetin on CYP1A1 expression or chemopreventive effects of human breast carcinoma MCF-7 remains unclear.

In the present study, we investigated the action of formononetin with the carcinogen activation pathway that is mediated by the AhR in MCF-7 cells.

MATERIALS AND METHODS

Materials. Formononetin (>99%) and charchol-dextran were obtained from sigma (St, Louis, Mo, USA). [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin purchased from ChemSyn. 7-ethoxyresorufin and resorufin was supplied by Pierce Chemical Co. 7,12-dimethylbenz[a]anthracene purchased from Chemsyn Science Lab. RPMI 1640, fetal bovine serum, penicillin-streptomycin solution, and trypsin were obtained from Life Technologies, Inc. Liquid scintillation cocktail was provided by Fisher.

Cell culture and treatment. The MCF-7 cells (American Type Culture Collection, Manassas, VA, USA) 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 otherwise indicated, all the experiments were carried out using confluent cells in a growth medium. Both formononetin and DMBA were dissolved in dimethylsulfoxide (DMSO). Stock solutions of these chemicals were added directly to the culture media and incubated with formononetin and DMBA. The control cells were treated with the DMSO only, and the final concentration of the solvent was always<0.2%.

7-Ethoxyresorufin-O-deethylase assay. The MCF-7 cells in 48-well plates were treated with formononetin at the concentrations 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. The fluorescence was measured for 30 min at an excitation wavelength of 530 nm and an emission wavelength of 590 nm in a FL-600 multi plate fluorescence reader (BIO-TEK, USA).

RNA preparation and CYP1A1 mRNA analysis by RT-PCR. The confluent MCF-7 cells were treated with formononetin and/or 1 μM of DMBA in growth medium for 6 h. The cells were washed twice with PBS, and the total RNA was isolated using the method previously 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 *et al.*, 1997). The cDNA was synthesized from 0.2 mg 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.

Transfection and reporter assay. CYP1A1-XRE-driven luciferase reporter plasmid (-1306 to -824 of the murine CYP1A1) (Jeong and Kim, 2000) containing four XRE was used to examine the specific activation of XRE. The MCF-7 cells were co-transfected transiently with 0.2 μg of pCMV-β-gal and 1 μg of XRE-regulated luciferase reporter plasmid (CYP1A1-XRE-Luc) per well using LipofectAMINE Plus. Four hour later, the medium was removed, and the cells were treated with or without DMBA (1 μM) and formononetin (10~50 μM). After exposing the cells for 18 h, they were washed with PBS and lysed. The supernatants were assayed for their luciferase and β-galactosidase activity. The luciferase activity was determined using a luciferase assay system (Promega) in accordance to the manufacturer's instructions using a luminometer (Luminoscan Ascent, Thermo electron Co.). The β-galactosidase assay was carried out as described elsewhere (Habig *et al.*, 1974). The luciferase activity was normalized to the β-galactosidase activity and is expressed as a proportion of the activity detected in the vehicle controls.

Immunocytochemistry. MCF-7 cells grown on poly-L-lysine-coated coverslips were treated with 50 μM formononetin and/or DMBA for 3 h, after which the cells were washed with PBS and fixed with 2% (w/v) paraformaldehyde. After permeabilization, coverslips were blockd with 1% BSA, followed by incubation with the goat anti-AhR polyclonal antibody (1 : 500 dilutions, Santa Cruz biotechnology) for 1 h. The secondary antibody was Alexa Fluor 488-conjugated donkey anti-goat antibody (1 : 500 dilutions, Molecular Probes) for 30 min. After two further washes in PBS, the sections were counterstained with the DNA dye 4',6-dianidino-2-phenylindole (DAPI) at a concentration of 1 μg/ml for 5 min. The cells were finally mounted with a mounting medium (Dako, Hamburg, Germany) and analyzed by confocal laser scanning microscopy (Zeiss, Jena, Germany).

Preparation of cytosol. Male Sprague-Dawley rats (200~250 g) were exposed to a 12 h 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 pending use. The protein concentrations were measured according to the method described by Bradford (1976).

AhR ligand binding assay. The binding of formononetin to AhR in rat liver cytosolic extracts was measured by determining the ability of formononetin 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 formononetin or solvent (DMSO) alone. Samples were incubated with gentle rotation at 4°C for 2 h. The unbound ^{3}H-labelled compounds were removed by adding 50 μl of a charcoal suspension (3% charcoal in 0.3% Dextran T-70), followed by incubation at 4°C for 1 h. 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. Using a microcentrifuge, the tubes were centrifuged at 3500 rpm for 5 min in a microcentrifuge. The HAP pellet was washed for an additional three times with 1 ml of HEDGK containing 0.5% Tween 80. After the last wash, 1 ml of absolute ethanol was added to the HAP pellet and the HAP/ethanol suspension was transferred to a scintillation vial. The tube and pipette tip were washed with an additional 0.5 ml ethanol, which was also added to the scintillation vial. Liquid scintillation cocktail solution (3 ml) was added to each vial and the radioactivity was quantified by liquid scintillation counting.

Measurement of DMBA-DNA adduct formation. Confluent cultures of MCF-7 cells in six-well plates were exposed to 0.1 μg/ml of [^{3}H]DMBA in the presence or absence of formononetin for 16 h. The cells were washed twice with cold PBS, and then trypsinized and pelleted. The nuclei were separated by incubating the cells for 10 min on ice in a lysis buffer A (10 mM-Tris-HCl pH 7.5, 320 mM-sucrose, 5 mM-magnesium chloride and 1% Triton X-100). The nuclei were collected by centrifugation at 5000 rpm for 10 min at 4°C after incubation. The nuclei were then lysed by adding 400 μl lysis buffer B (1% SDS in 0.5 M-Tris, 20 mM-EDTA and 10 mM-NaCl, pH 9), followed by a treatment with 20 μl proteinase K (20 mg/ml) for 2 h at 48°C. The samples were then allowed to cool at room temperature, and the residual protein was salted out by adding 150 μl saturated NaCl. The samples were centrifuged at 13000 rpm for 30 min at 4°C. The genomic DNA was isolated from the supernatant fraction by ethanol precipitation and reconstituted in autoclaved water. The amount and

purity of the extracted DNA was determined by measuring the absorbance at 260 nm/or 280 nm.

Statistical analysis. All experiments were repeated at least three times to ensure reproducibility. The results are reported as a mean ± SD 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

Effects of formononetin on CYP1A1 activity. The CYP1A1 gene activity in MCF-7 cells treated with formononetin with or without DMBA was measured using a CYP1A1-specific EROD activity assay. The formononetin (10~50 μM) alone significantly induced the EROD activity in a dose dependant manner (Fig. 1A). Also, the formononetin significantly decreased the DMBA-induced EROD activity in a dose dependent manner (Fig. 1B). The formononetin-mediated suppression of EROD induction was not the result of a formononetin cytotoxic effect (data not shown).

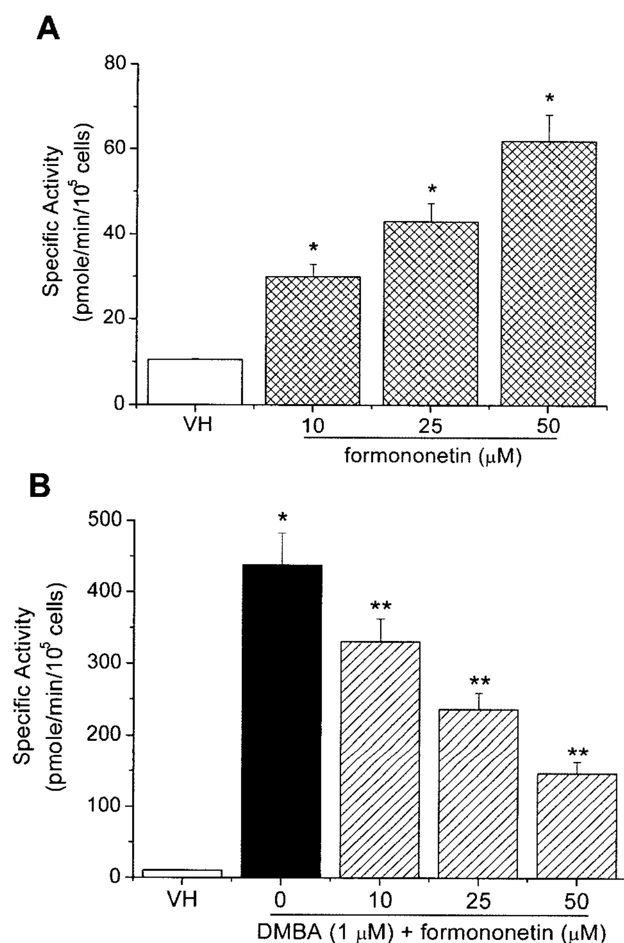


Fig. 1. Effects of formononetin on the EROD activity in MCF-7 cells. The cells were treated with DMSO (VH), formononetin (10 μM~50 μM: A), DMBA (1 μM) plus formononetin (10 μM~50 μM: B) for 18 h, as described in Materials and Methods. The values are reported as a mean ± SD of triplicate cultures. *P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

Effects of formononetin on CYP1A1 mRNA. The CYP1A1 mRNA in MCF-7 cells treated with DMBA in the presence or absence of formononetin was measured using RT-PCR. Treatment with formononetin resulted in a dose dependent increase in CYP1A1 mRNA accumulation from 10 to 50 μM (Fig. 2A). Also, treating the cells with 1 μM DMBA for 6 h increased the CYP1A1 mRNA accumulation. The formononetin inhibited the DMBA-induced CYP1A1 mRNA in a dose dependant manner (Fig. 2B).

Effects of formononetin on XRE luciferase activity. The effect of formononetin on the transactivation of XRE reporter gene (pCYP1A1-XRE-Luc) was assessed in order to determine if formononetin causes the transcriptional inhibition of the CYP1A1 genes through AhR activation. The cells were treated with DMBA and/or formononetin and the XRE-driven luciferase activities were determined. The formononetin alone significantly induced the XRE-driven luciferase activity in a dose dependant

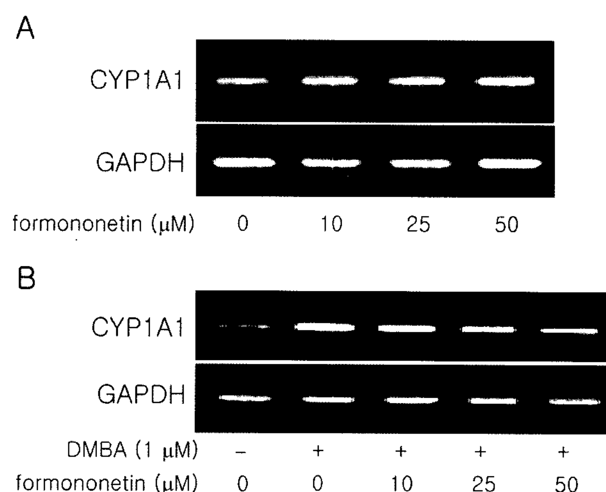


Fig. 2. Effects of formononetin on CYP1A1 mRNA by RT-PCR analysis. The MCF-7 cells were treated with DMSO (VH), formononetin (10 μM~50 μM: A), DMBA (1 μM) plus formononetin (10 μM~50 μM: B) for 6 h. The total cellular RNA was isolated from the cells. The PCR amplification products were electrophoresed in 2% agarose gel and stained with ethidium bromide.

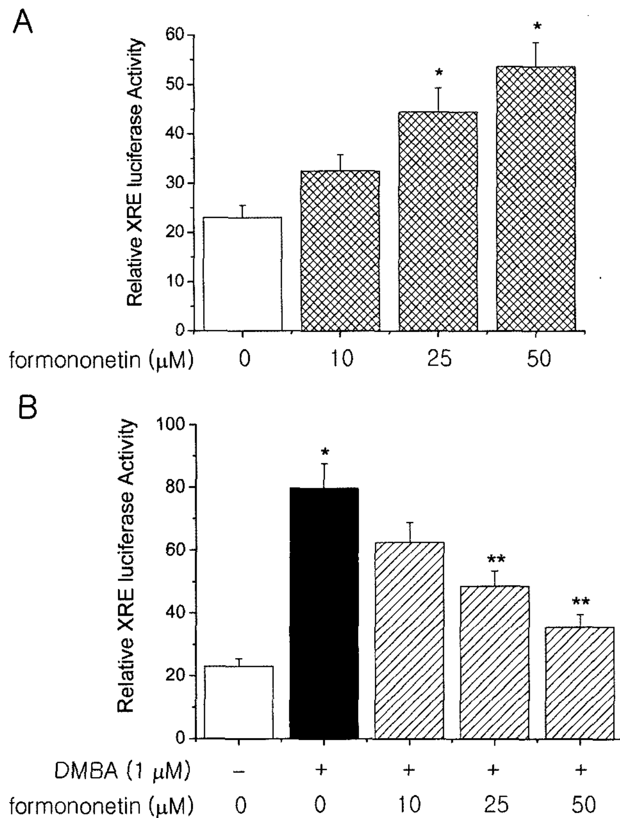


Fig. 3. Effects of formononetin on DMBA-induced AhR transactivation by XRE reporter assay. The MCF-7 cells were transient transfected with the XRE-Luc reporter plasmids, and treated with DMSO (VH), formononetin (10 μM~50 μM: A), DMBA (1 μM) plus formononetin (10 μM~50 μM: B). After 18 h treatment, the cells were harvested, and luciferase activities were determined. The values are reported as the mean ± SD of triplicate cultures. *$P < 0.01$ significantly different from the VH. **$P < 0.01$ significantly different from DMBA.

manner (Fig. 3A). Also, the DMBA treatment caused an increase in the luciferase activity compared to the control. However, when the cells were simultaneously treated with DMBA and formononetin, the DMBA-induced XRE-driven luciferase activity was significantly suppressed by formononetin (10~50 μM) in a dose-dependent manner (Fig. 3B).

Effect of formononetin on binding of TCDD to AhR. The EROD and CYP1A1 gene expression data suggest that formononetin is a ligand for the AhR. This was further demonstrated by examining the ability of formononetin to compete with the prototypical AhR ligand TCDD for binding to the AhR in the cytosolic fraction isolated from rats. As shown in Fig. 4, formononetin (10~50 μM) competitively inhibited specific [^{3}H]TCDD binding to AhR.

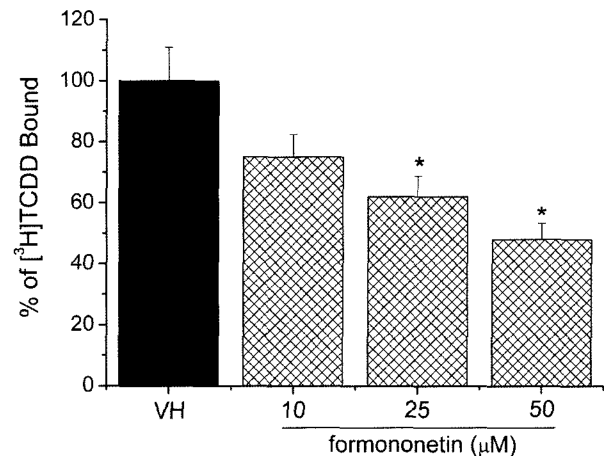


Fig. 4. Effects of formononetin on [^{3}H]TCDD binding to cytosolic AhR. Cytosolic extracts (3 mg/ml) containing AhR were incubated for 2 h at 4°C with 10 nM [^{3}H]TCDD or DMSO. The percentage of [^{3}H]TCDD bound for formononetin in the competition assay was calculated by dividing the disintegrations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

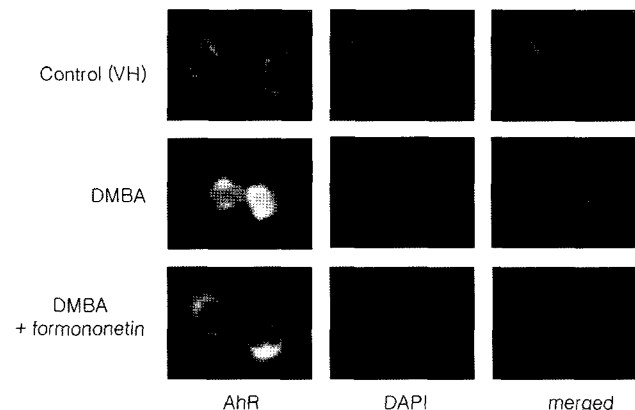


Fig. 5. Effects of formononetin on DMBA-induced AhR nuclear translocation. The MCF-7 cells were treated with DMSO (VH), DMBA (1 μM) plus formononetin (50 μM) for 3 h. Cells were fixed and subjected to immunocytochemistry using an AhR antibody (left column), the images were superimposed with DAPI stained images (middle column) and merged images (right column).

Effect of formononetin on AhR nuclear translocation. Next we examined whether formononetin regulates the nuclear translocation of AhR in MCF-7 cells followed by detection using fluorescence microscopy. In control cells were AhR protein localized in the cytoplasm, however, after treatment with DMBA for 3 h GFP fluorescence was found almost exclusively in the

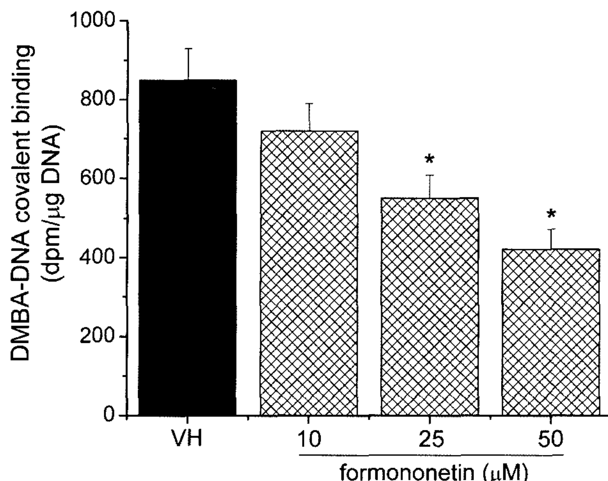


Fig. 6. Effects of formononetin on the formation of the [3H]DMBA-DNA adducts in MCF-7 cells. The cells were seeded in 6-well plates and treated with [3H]DMBA or [3H]DMBA plus formononetin (10 μM~50 μM). After 16 h of treatment, the genomic DNA was extracted and the [3H]DMBA-DNA lesions were determined by scintillation counting. The values are reported as the mean ± SD of triplicate cultures. *$P < 0.01$ significantly different from the positive control (DMBA).

nucleus. Treatment of the cells with formononetin (50 μM) during DMBA exposure resulted in inhibition of AhR nucleus translocation (Fig. 5).

Effect of formononetin on metabolic activation of DMBA. The metabolic activation of DMBA by CYP1A1 leads to metabolites that react specifically with DNA. The MCF-7 cells were incubated with [3H]DMBA in the presence or absence of formononetin for 16 h, and the catabolism of DMBA was determined by scintillation counting. Exposing the cells to [3H]DMBA in the presence of formononetin (10~50 μM) inhibited the formation of the DMBA-DNA adduct in a dose dependent manner (Fig. 6).

DISCUSSION

Several isoflavones have shown their anti-carcinogenic effects in various models. Isoflavones are abundant in legumes such as soy, mung bean sprouts, kidney beans, navy beans, red clover, and Japanese arrowroot (Mazur *et al.*, 1998; Horn-Ross *et al.*, 2000). Both protective and adverse health effects have been associated with consumption of foods and food supplements containing isoflavone derivatives. Dietary consumption of isoflavones has been linked to reduce the risks to breast cancer (Lamartiniere *et al.*, 1998) and prostate cancer (Adlercreutz *et al.*, 1995). A number of naturally occurring isoflavones have been shown to modulate the CYP450 system, which could result on the activation or inhibition of these enzymes. On other hand, some isoflavones may alter the CYPs by binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor and acts either AhR agonists or antagonists. Phytoestrogen formononetin (4-methoxy-7-hydroxyisoflavone) are the 4-*O*-methyl derivatives of daidzein (4,7-dihydroxyisoflavone), and are the predominant isoflavones found in alfalfa (Horn-Ross *et al.*, 2000), red clover (Mazur *et al.*, 1998), and chick peas (Mazur *et al.*, 1998). Thus far, there are no reports of its effect on the AhR, CYP1A1 mRNA accumulation and CYP1A1 enzymatic activity. Therefore, in this study we investigated the action of formononetin with the carcinogen activation pathway that is mediated by the AhR in MCF-7 breast carcinoma cells. We examined the effect of formononetin on the AhR and the major carcinogen-activating enzyme in MCF-7 cells as well as CYP1A1. The treatment with formononetin alone significantly induced the EROD activity. DMBA induced the CYP1A1 activity as measured by the EROD activity. However, treatment with formononetin during DMBA exposure resulted in a dose dependent inhibition of CYP1A1 activity. Surprisingly, treating the MCF-7 cells with formononetin in the absence of DMBA resulted in enhancement of CYP1A1 mRNA. Using RT-PCR, we found that the mammary carcinogen DMBA elevated the CYP1A1 mRNA level in MCF-7 cells that was partially antagonized by simultaneous treatment of the cells with formonetin. It has been shown previously that the ability of the liganded AhR to induce transcription of genes depends on its ability to bind enhancer sequences, called the XRE, flanking the 5'-promoter region of CYP1A1. To determine whether formononetin 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 formononetin. Specific binding was separated from non-specific binding by hydroxyapatite chromatography. Formononetin partially inhibited TCDD binding to the AhR. Thus, formononetin appears to be an antagonist of the AhR in the presence of other AhR ligands including DMBA or TCDD. We next examined whether formononetin regulates of nuclear translocation of AhR in MCF-7 cells, followed by detection using fluorescence microscopy. In control cells were AhR protein localized in the cytoplasm. However, after treatment with DMBA for 3 h, GFP fluorescence was found almost exclusively in the nucleus. Treatment of the cells with formononetin during DMBA exposure resulted inhibition of AhR nucleus

translocation. These results suggest that formononetin reduces the DMBA-inducible subsequent nuclear translocation AhR. Furthermore, inhibition of the AhR-mediated response of CYP1A1 to DMBA and direct inhibition of CYP1A1 activity by formononetin would be expected to reduce the metabolic activation of DMBA. The formation of adducts between DNA and the reactive metabolites of DMBA, which result from CYP1A1 activity, were reduced substantially in formononetin-treated MCF-7 cells. Exposing the cells to [^{3}H]DMBA in the presence of formononetin inhibited DMBA-DNA adduct formation in a dose dependent manner.

The current data suggested that formononetin may be involved in the chemopreventive properties, by reducing the formation of carcinogens through inhibition of enzyme, such as CYP1A1, a well known model in carcinogen activation. However, the fact that formononetin induces CYP1A1 *via* the AhR and is an inhibitor of CYP1A1 activity suggests that it might be a natural ligand for AhR. 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). Formononetin has an important chemopreventive feature that might be applied to a wide range of procarcinogens. One advantage of formononetin as chemopreventive agents in human trials is that, unlike synthetic chemopreventive agents, it is a naturally occurring compound produced endogenously by plants. Therefore, formononetin represents a new class of relatively non-toxic anti-tumorigenic AhR agonists which are of phytochemical origin.

ACKNOWLEDGMENTS

This work was supported by Korea Research Foundation Grant (KRF-2004-041-C00446).

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