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

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Phytoestrogen biochanin A is an isoflavone derivative isolated from red clover *Trifolium pratense* with anticarcinogenic properties. This study examined the action of biochanin A with the carcinogen activation pathway that is mediated by the aryl hydrocarbon receptor (AhR) in MCF-7 breast carcinoma cells. Treating the cells with biochanin A alone caused the accumulation of CYP1A1 mRNA and an increase in CYP1A1-specific 7-ethoxyresorufin *O*-deethylase (EROD) activity in a dose dependent manner. A concomitant treatment with 7,12-dimethylbenz[a]anthracene (DMBA) and biochanin A markedly reduced the DMBA-inducible EROD activity and CYP1A1 mRNA level. In addition, the biochanin A treatment alone activated the DNA-binding capacity of the AhR for the dioxin-response element (DRE) of CYP1A1, as measured by the electrophoretic-mobility shift assay (EMSA). EMSA revealed that biochanin A reduced the level of the DMBA-inducible AhR-DRE binding complex. Furthermore, biochanin A competed with the prototypical AhR ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), for binding to the AhR in an isolated rat cytosol. The biochanin A competitively inhibited the metabolic activation of DMBA, as measured by the formation of the DMBA-DNA adducts. These results suggest that biochanin A may thus be a natural ligand to bind on AhR. Therefore, biochanin A may be due to act an antagonist/agonist of the AhR pathway.

Key words: Biochanin A, 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. 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 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 (Gonzalez, 1990; 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*

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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).

Phytoestrogen biochanin A is an isoflavone derivative isolated from red clover *Trifolium pratense* with anti-carcinogenic properties. Phytoestrogens from the isoflavone family are found in several plants used for human and animal nutrition (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; Fustier *et al.*, 2003). Biochanin A is a methyl derivative of genistein, and has been reported to protect against mammary carcinogenesis in rats (Gotoh *et al.*, 1998), and mammary tumor virus-induced spontaneous breast cancer in mice (Mizunuma *et al.*, 2002). Biochanin A has also been reported to reduce the xenobiotic-induced CYP1A1 and CYP1B1 abundance through an interference of DRE-dependent transactivation in breast cancer MCF-7 cells (Chan *et al.*, 2003). In the present study, we have used the human mammary epithelial carcinoma MCF-7 cell line to examine the interaction between biochanin A and the AhR pathway. We report, for the first time, that biochanin A also to induce accumulation of CYP1A1 mRNA and induce CYP1A1 enzymatic activity by activation of the AhR. Biochanin A 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 biochanin A 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 biochanin A with the carcinogen activation pathway that is mediated by the AhR in MCF-7 cells.

MATERIALS AND METHODS

Materials

All chemicals and cell culture materials were obtained from the following sources: biochanin A (>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 Technologies, Inc); Liquid scintillation cocktail (Fisher); charcoal-dextran (Sigma).

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 biochanin A and DMBA were dissolved in dimethyl-sulfoxide (DMSO). Stock solutions of these chemicals were added directly to the culture media and incubated with biochanin A 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 biochanin A at the concentrations indicated in the figures with or without 1 μM of DMBA in a growth medium for 18 h. After incubation, the medium was removed and the wells were washed twice with fresh medium. The 7-Ethoxy-resorufin-*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.

RNA preparation and CYP1A1 mRNA analysis by RT-PCR

The confluent MCF-7 cells were treated with biochanin A 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 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.

Electrophoretic mobility shift analysis

Confluent cultures of MCF-7 cells were treated with 2 μM of DMBA and/or biochanin A (10~50 μM). The nuclear extracts were prepared and EMSA was performed according to the procedure reported elsewhere (Jeong, 1997). Synthetic DNA oligonucleotides containing the AhR-binding site of the DRE (Chen and Tukey, 1996) were labeled with [^{32}P]ATP. The nuclear extract (5 μL/10 μg protein) was generally mixed with 15 μL HEDG, containing 1 mM dithiothreitol and 0.1 mM PMSF, and 1.0 μg of poly (dI·dC), and incubated for 20 min at 20°C prior to adding 1 μL of ^{32}P-labeled synthetic oligonucleotide

(100,000 dpm). After incubation for an additional 20 min, the samples were run on a 4% polyacrylamide gel with a recirculating 1×TAE buffer (6.7 mM Tris–HCl containing 3.3 mM sodium acetate and 1.0 mM EDTA, pH 8.0). The gel was dried and exposed at -80°C to X-ray film.

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 until use. The protein concentrations were measured using the Bradford method (1976) with bovine serum albumin as the standard and adjusted to 10-15 mg/mL diluted in HEDGK buffer.

AhR ligand binding assay

The binding of biochanin A to AhR in rat liver cytosolic extracts was measured by determining the ability of biochanin A 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 biochanin A 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. The tubes were centrifuged at 3500 rpm and 4°C for 5 min in a microcentrifuge. The HAP pellet was washed 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 of ethanol, which was also added to the scintillation vial. Liquid scintillation cocktail solution (5 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 biochanin A 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 10min 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 to 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 redissolved in autoclaved water. The amount and purity of the extracted DNA was determined by measuring the absorbance at 260 nm/or 280 nm (Miller *et al,* 1988).

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

Effects of biochanin A on CYP1A1 activity

The CYP1A1 gene activity in MCF-7 cells treated with biochanin A 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 h, there was an increase in the CYP1A1 enzyme activity, as measured by EROD assay. Biochanin A alone significantly induced the EROD activity in a dose dependant manner (Fig. 1A). Biochanin A significantly decreased the DMBA-induced EROD activity in a dose dependent manner (Fig. 1B). The biochanin A-mediated suppression of EROD induction was not the result of a biochanin A cytotoxic effect (data not shown).

Effects of biochanin A on CYP1A1 mRNA

The CYP1A1 mRNA in MCF-7 cells treated with DMBA in the presence or absence of biochanin A was measured using RT-PCR. Surprisingly, the biochanin A treatment alone caused an approximately 3.5-fold increase in the CYP1A1 mRNA level. Treatment with biochanin A 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 h caused a 5.5-fold increase in CYP1A1 mRNA accumulation. Biochanin A inhibited the

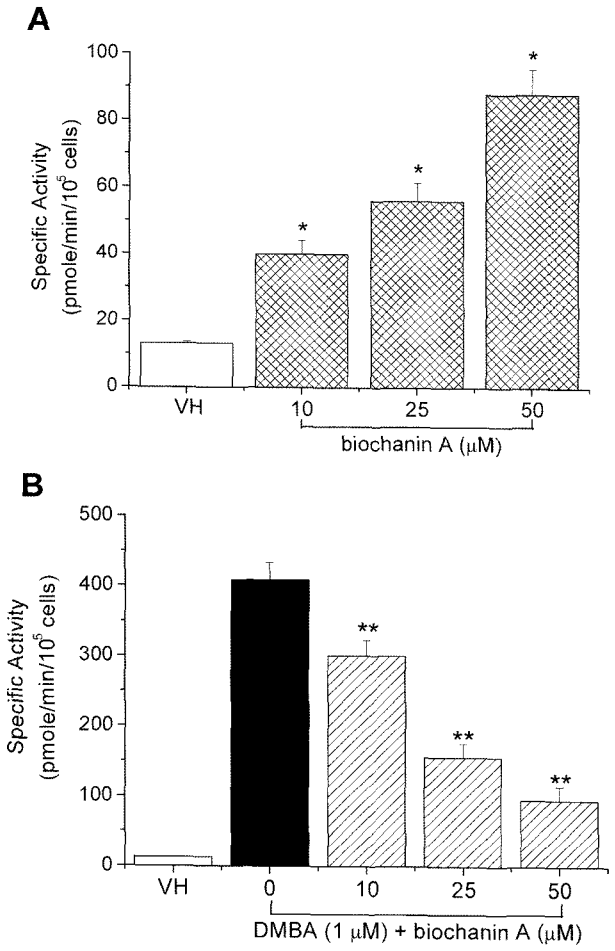

Fig. 1. Effects of biochanin A on the EROD activity in MCF-7 cells. The cells were treated with DMSO (VH), biochanin A (10 μM~50 μM: A) or DMBA (1 μM) plus biochanin A (10 μM~50 μM: B) for 18 h, as described in Materials and Methods. The values are reported as a mean ± S.D. of triplicate cultures. *P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

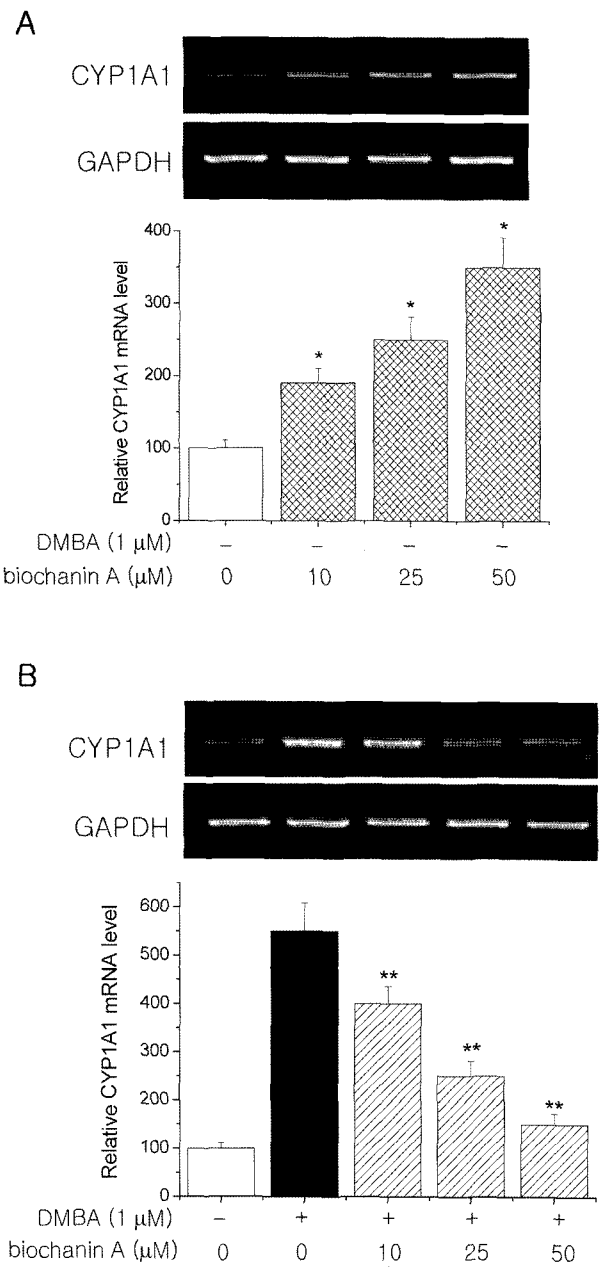

Fig. 2. RT-PCR analysis of CYP1A1 mRNA in MCF-7 cells. The cells were treated with DMSO (VH), biochanin A (10 μM~50 μM: A) or DMBA (1 μM) plus biochanin A (10 μM~50 μM: B) for 6 h. The total cellular RNA was isolated from the cells. The PCR amplification products were electrophoresed in 2% agarose gel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

DMBA-induced CYP1A1 mRNA in a dose dependant manner (Fig. 2B).

Activation of the AhR DNA-binding activity by biochanin A

The effect of biochanin A on the nuclear accumulation of activated AhR induced by DMBA was examined using an electrophoretic mobility shift assay (EMSA) on the nuclear extracts of the MCF-7 cells that had been treated with biochanin A in the presence or absence of DMBA. Biochanin A only at 50 μM caused an increase in activated AhR (Fig. 3A). In addition, DMBA at 1 μM caused an increase in the DNA-binding ability of AhR for an oligonucleotide containing the DRE compared with the control. Biochanin A reduced the extent of the DMBA-induced transformation of an AhR/^{32}P-DRE complex (Fig. 3B). The specificity of this interaction was verified by the ability of a 200-fold excess of an unlabeled DRE oligonucleotide to compete with the DMBA-induced binding of the transformed AhR to a ^{32}P-DRE.

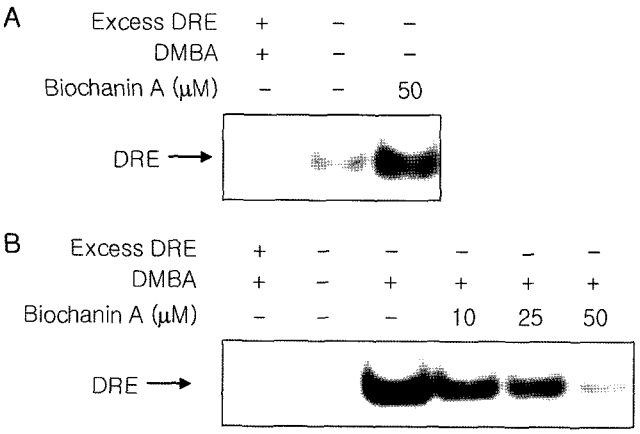

Fig. 3. *Electrophoretic mobility shift assay of the DRE-binding proteins* in MCF-7 cells. The cells were treated with DMSO (VH), biochanin A (50 μM: A) or DMBA (1 μM) plus biochanin A (10 μM~50 μM: B) for 1 h. The nuclear extracts were isolated and used in the EMSA with the ^{32}P-labeled DRE oligonucleotide as a probe, as described in Materials and Methods. The arrow indicates the AhR-DRE complex. Excess DRE; a 200-fold excess of nonlabeled DRE.

Effect of biochanin A on binding of TCDD to AhR

The EROD and CYP1A1 gene expression data suggests that biochanin A is a ligand for the AhR. This was further demonstrated by examining the ability of biochanin A 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, biochanin A competitively inhibited specific [^{3}H]TCDD binding to AhR by approximately 62%.

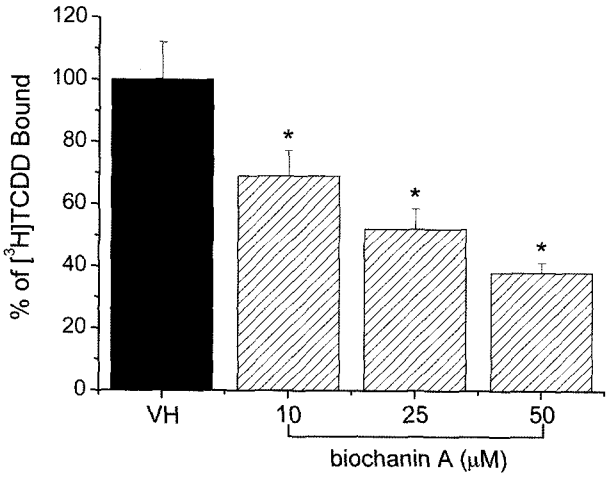

Fig. 4. Biochanin A competes with [^{3}H]TCDD for binding to cytosolic AhR. Cytosolic extracts (3 mg/mL) containing AhR were incubated for 2 h at 4°C with 10 nM [^{3}H]TCDD or DMSO. The percentage of [^{3}H]TCDD bound for biochanin A in the competition assay was calculated by dividing the disintegrations per minute of specific [^{3}H]TCDD bound in the biochanin A 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 (VH).

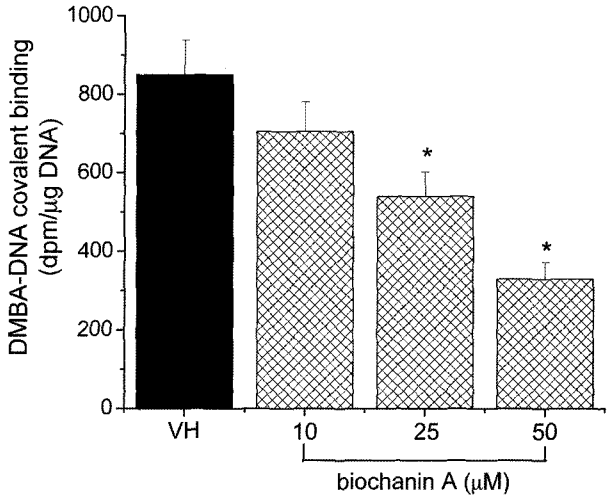

Fig. 5. *Effects of biochanin A on the formation of the* [^{3}H]DMBA-DNA adducts in MCF-7 cells. The cells were seeded in 6-well plates and treated with [^{3}H]DMBA or [^{3}H]DMBA plus biochanin A (10 μM~50 μM). After 16 hr of treatment, the genomic DNA was extracted and the [^{3}H]DMBA-DNA lesions were determined by scintillation counting. The values are reported as the mean ± S.D. of triplicate cultures. *P <0.01 significantly different from VH (DMBA).

These data demonstrate that biochanin A can compete directly binding to AhR.

Effect of biochanin A 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 [^{3}H]DMBA in the presence or absence of biochanin A for 16 h, and the metabolism of DMBA was determined by scintillation counting. Exposing the cells to [^{3}H]DMBA in the presence of biochanin A inhibited the formation of the DMBA-DNA adduct in a dose dependent manner (Fig. 5).

DISCUSSION

Several flavonoids have shown their anti-carcinogenic effects in various models. The red clover (Trifolium pratense) isoflavone biochanin A is a methylated derivative of genistein, and its anti-mutagenic effect in bacterial cells has been shown previously (Chan *et al.*, 2003). 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. Biochanin A is of particular interest because it affects other *in vitro* mechanisms that are relevant to chemoprevention (Chan *et al*, 2003).

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 biochanin A with the carcinogen activation pathway that is mediated by the AhR in MCF-7 breast carcinoma cells. We examined the effect of biochanin A on the AhR and the major carcinogen-activating enzyme in MCF-7 cells, CYP1A1. The treatment with biochanin A 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 biochanin A during DMBA exposure resulted in the dose dependent inhibition of CYP1A1 activity (Fig. 1B). Surprisingly, treating the MCF-7 cells with biochanin A 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 biochanin A (Fig. 2A). 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. 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 biochanin A (Fig. 2B). We performed EMSA to determine whether biochanin A was capable of regulate the transformation of AhR that are capable of specific binding to ^{32}P-labeled double-stranded oligonucleotides containing DRE sequence. Using EMSA to measure the nuclear accumulation of activated AhR, we showed that biochanin A could partially inhibit the activation of AhR accounts for the decrease in DMBA-induced CYP1A1 mRNA seen in Fig. 2A. After treatment of DMBA and/or biochanin A with MCF-7 cells, nuclear extracts were isolated and then performed EMSA. Treatment with biochanin A alone induced AhR/^{32}P-DRE complex (Fig. 3A). However, biochanin A reduced the DMBA-induced transformation of an AhR/^{32}P-DRE complex (Fig. 3B). The specificity of this interaction was verified by the ability of a 200-fold excess of unlabeled DRE oligonucleotide to compete away the DMBA-induced binding of the transformed AhR to a ^{32}P-DRE. Biochanin A, therefore, activates the AhR, induces CYP1A1 mRNA accumulation, and induces CYP1A1 enzymatic activity. These results suggest but do not prove conclusively that biochanin A is a ligand of the AhR. To determine whether biochanin A 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 biochanin A. Specific binding was separated from nonspecific binding by hydroxyapatite chromatography. As shown in Fig. 4, biochanin A partially inhibited TCDD binding to the AhR. It seems likely that the inhibition of DMBA-induced AhR activation by biochanin A demonstrated in the EMSA in Fig. 3B. Thus, biochanin A 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 biochanin A 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 biochanin A-treated MCF-7 cells. Exposing the cells to [^{3}H]DMBA in the presence of biochanin A inhibited DMBA-DNA adduct formation in a dose dependent manner (Fig. 5).

The current data suggested that biochanin A 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 biochanin A induces CYP1A1 *via* the AhR and is a 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 (McDougal *et al.*, 1997; Krishnan *et al.*, 1995). Therefore, biochanin A represents a new class of relatively non-toxic anti-tumorigenic AhR agonists which are of phytochemical origin.

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