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The red clover (*Trifolium pratense*) isoflavone biochanin A modulates the biotransformation pathways of 7,12-dimethylbenz[a]anthracene

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Several flavonoids have shown their anti-carcinogenic effects in various models. The soyabean isoflavone genistein was demonstrated earlier in our laboratory to be an effective inhibitor of dimethylbenz[a]anthracene (DMBA)-induced DNA damage in MCF-7 cells by curbing cytochrome P450 (CYP) 1 enzymes. 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. Because of its protection against chemical carcinogenesis in an animal model, biochanin A was selected for testing in our established MCF-7 cell system. From the results obtained in the semi-quantitative reverse transcription–polymerase chain reaction and xenobiotic response element (XRE)–luciferase reporter assays, biochanin A could reduce xenobiotic-induced CYP1A1 and -1B1 mRNA abundances through the interference of XRE-dependent transactivation. Enzyme kinetic studies also indicated that biochanin A inhibited both CYP1A1 and -1B1 enzymes with inhibition constant (K_i) values 4·00 and 0·59 μM respectively. Since the biotransformation of DMBA was dependent on CYP1 enzyme activities, biochanin A was able to decrease the DMBA–DNA lesions. The present study illustrated that the red clover isoflavone could protect against polycylic aromatic hydrocarbon-induced DNA damage.

Biochanin A: Cytochrome P450 1A1: Cytochrome P450 1B1: 7,12-Dimethylbenz[a]anthracene–DNA lesion

Breast cancer is one of the most common cancers in women and is responsible for 20 % of all female cancers (Higginson *et al.* 1992). From the migration pattern and breast-cancer incidence, environment has been demonstrated to be a factor in the aetiology of breast-cancer. Asian women have lower breast-cancer incidence than those in the West; however, the breast-cancer risk increased by 80 % for oriental female immigrants resident for >10 years in the USA (Ziegler *et al.* 1993).

Polycyclic aromatic hydrocarbons (PAH) are commonly found in diesel exhaust, char-broiled meat, tobacco smoke, over-heated cooking oil, etc. (International Agency for Research on Cancer, 1983; Environmental Protection Agency, 1990). These toxic compounds are metabolized into DNA-attacking species in man, and increased amount of PAH–DNA adducts were discovered in human breast tumours (Li *et al.* 1996). Therefore, interrupting PAH metabolism could reduce the risk of breast cancer.

The transformation of PAH to their DNA-attacking configurations can be affected by aryl hydrocarbon receptor (AHR) through the gene expression regulation of xenobiotic metabolizing enzymes. The activated AHR translocates to the nucleus and heterodimerizes with AHR nuclear translocator. The dimers initiate the transcriptional activities of xenobiotic-responsive genes (Kronenberg *et al.* 2000). Cytochrome P450 (CYP) 1 family genes, which are responsible for the biotransformation of PAH, can be regulated in this pathway, (Dertinger *et al.* 2000; Safe, 2001). The significance of AHR and CYP1 enzymes in PAH-induced carcinogenesis is demonstrated in AHR null mice (Shimizu *et al.* 2000) and CYP1B1 knockout mice (Buters *et al.* 1999). The inhibition of CYP1 enzymes appears to be beneficial in the prevention of 7,12-dimethylbenz[a]anthracene (DMBA)–DNA adduct formation *in vivo* and *in vitro* (MacDonald *et al.* 2001; Kleiner *et al.* 2002). In epidemiological studies, the polymorphisms of increased expressions of

Abbreviations: AHR, aryl hydrocarbon receptor; CYP, cytochrome P450; DMBA, 7,12-dimethyl benz[a]anthracene; EROD, ethoxyresorufin-*O*-deethylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; XRE, xenobiotic response element.

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cyp1a1 (Taioli, 1999) and *cyp1b1* (Zheng *et al.* 2000) are risk factors for breast cancer.

Isoflavones have been suggested to be anticarcinogenic agents, and genistein has been one of the most studied isoflavones. Biochanin A isolated from red clover (*Trifolium pratense*) is a methyl derivative of genistein. Many studies have documented its chemopreventive effects. Biochanin A can protect against nitrosomethylurea-induced mammary carcinogenesis in rats (Gotoh *et al.* 1998), and mammary tumour virus-induced spontaneous breast cancer in mice (Mizunuma *et al.* 2002). It is also growth-inhibitory to human mammary epithelial cells and breast cancer MCF-7 cells (Peterson *et al.* 1996). Because of the protection against chemical-induced mammary carcinogenesis in rats and the relationship between PAH and breast cancer, we wanted to investigate the effect of biochanin A on the PAH metabolizing enzymes CYP1A1 and -1B1 in the current study.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

MCF-7 cells (a gift from Dr Craig Jordan, Northwestern University, IL, USA) were cultured in Roswell Park Memorial Institute-1640 Phenol Red-free media (Sigma-Aldrich) and fetal bovine serum (100 ml/l; Invitrogen Life Technologies, Rockville, MD, USA) at 37°C and 5 % CO_2. Sub-confluent cell cultures were treated with 1 μM-DMBA and 0·1–10·0 μM-biochanin A for 24 h. In order to maintain consistency, the cell density was maintained at 5×10^2 cells/mm^2 for all experiments.

*Ethoxyresorufin-*O*-deethylase activities in intact MCF-7 cells*

The assay method was performed as previously described (Ciolino & Yeh, 1999) with some modifications. In brief, MCF-7 cells cultured in ninety-six-well plates were treated with 1 μM-DMBA and various concentrations of biochanin A. DMBA and biochanin A were dissolved in dimethylsulfoxide and control received dimethylsulfoxide. The amount of dimethylsulfoxide was standardized to 1 ml/l in all cases. The medium was then removed and the cells were washed twice with 100 μl PBS. Ethoxyresorufin-*O*-deethylase (EROD) activity assays (an indicator for both CYP1A1 and -1B1) were then carried out. Ethoxyresorufin (50 μl, 5 μmol/l PBS) with 1·5 mM-salicyclamide was added in each well, and incubated at 37°C for 15 min. The reaction was stopped by 50 μl ice-cold methanol, and the resorufin generated was measured by a FLUOstar Galaxy microplate reader (BMG Labtechnologies, Offenburg, Germany) with an excitation of 544 nm and emission at 590 nm. The activities were quantified against resorufin standards. In order to avoid misinterpretation caused by differences in viable cells, an equivalent set of cultured cells was used for determining cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The EROD activity was then normalized with the cell viability.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cell viability was assessed by MTT staining as described by Mosmann (1983). Briefly, MCF-7 cells were plated and cultured as described. At the end of the treatment, 50 μl MTT (1 mg/ml) was added and the cells were incubated at 37°C for 4 h. The surviving cell content was determined by the absorbance at 600 nm.

Enzyme inhibition assays

Recombinant human CYP1A1 and -1B1 enzymes expressed in baculovirus-infected insect microsomes (Supersomes®) were purchased from Gentest Corp. (Woburn, MA, USA). Protein (2 p mol) was incubated in 100 μl PBS, pH 7·2 with 400 nM-ethoxyresorufin and biochanin A in different concentrations. The reaction was initiated by 500 μM-NADPH, and stopped by 100 μl ice-cold methanol after 20 min of incubation. The fluorescence was measured as described earlier.

Measurement of 7,12-dimethylbenz[a]anthracene–DNA adduct formation

This assay was performed as previously described (Ciolino *et al.* 1999). MCF-7 cells were plated in six-well plates at 5×10^5 cells per well and allowed to attach for 24 h. Then 0·1 μg [^{3}H]DMBA (Amersham, Arlington Height, IL, USA)/ml was administered with or without biochanin A. After 16 h, cells were washed twice with cold PBS, trypsinized and pelleted. Nuclei were separated by incubating the cells for 10 min on ice in lysis buffer A (10 mM-Tris-HCl pH 7·5, 320 mM-sucrose, 5 mM-magnesium chloride and Triton X-100 (10 ml/l)). The nuclei were collected by centrifugation at 5000 rpm for 10 min at 4°C after the incubation. The nuclei were then lysed by 400 μl lysis buffer B (SDS (10 ml/l) in 0·5 M-Tris, 20 mM-EDTA and 10 mM-NaCl, pH 9), followed by the treatment with 20 μl proteinase K (20 mg/ml) for 2 h at 48°C. After that, the samples were allowed to cool to room temperature and the residual protein was salted out by adding 150 μl saturated NaCl. The samples were then subjected to centrifugation at 13 000 rpm for 30 min at 4°C. Genomic DNA was isolated from the supernatant fraction by ethanol precipitation, and redissolved in autoclaved water. Absorbances at 260 and 280 nm were employed to determine the amount and purity of the extracted DNA. DNA samples attaining a 260:280 nm ratio $>1{\cdot}9$ were used for scintillation counting.

Xenobiotic response element–luciferase gene reporter assay

Cloning of xenobiotic response element-driven reporter gene. A fragment with five xenobiotic response elements

(XRE) from rat *cyp1a1* 5′-flanking region was amplified from rat genomic DNA as described by Backlund *et al.* (1997). No other response elements were identified in this fragment. The polymerase chain reaction (PCR) product was digested with SmaI and BamHI and subcloned into a firefly luciferase reporter vector pTA–Luc (Clontech, Palo Alto, CA, USA).

Dual luciferase assays. MCF-7 cells were seeded at 10^5 cells per well in twenty-four-well plates. After 24 h, the cells were transiently transfected with 4·0 μg XRE reporter plasmid and 1·0 μg renilla luciferase control vector pRL (Promega, Madison, WI, USA) in Lipofect-Amine (Invitrogen Life Technologies). After 16 h, the medium was removed and the cells were treated with 1 μM-DMBA and various concentrations of biochanin A for 24 h. The amounts of these two luciferases were determined using dual-luciferase-assay kit (Promega). The luciferase bioluminescence was measured by using a FLUOstar Galaxy plate reader (BMG Lab technologies). The XRE transactivation activities represented by firefly luciferase light units were then normalized by that of Renilla luciferase.

Semi-quantitative reverse transcription–polymerase chain reaction assay

A reverse transcription–PCR assay was used to quantify mRNA expression. Total RNA was isolated from cells grown in six-well plates (Costar Corning Inc., NY, USA) in triplicate by a method previously described (Wang & Phang, 1995). RNA (1 μg) was used for cDNA synthesis, and the final volume was diluted to 20 μl. Primers of CYP1A1, CYP1B1 and β-actin sequences as published previously (Dohr *et al.* 1995) and a Perkin Elmer thermocycler (GeneAmp PCR System 2400; Perkin Elmer, Norwalk, CT, USA) were utilized to amplify the target cDNA separately after the first strand reaction. All PCR reactions consisted of 0·2 mM-dNTP, 2 μl cDNA, 0·2 μmol each primer/l, 1 × PCR buffer and 1 U Taq polymerase (Invitrogen Life Technologies). The conditions were 94°C for 45 s, 65°C for 45 s, 72°C for 60 s and a final extension period of 7 min at 72°C. There were twenty-five amplification cycles for CYP1A1, twenty-three for CYP1B1 and nineteen for β actin. The PCR products were separated on agarose (1·8 %) gel, stained with ethidium bromide, and photographed. A scanner equipped with Scion Image software (Scion Corporation, Frederick, MD, USA) was used to compare the optical density of the amplified fragments. The linearity of signals was verified in separate experiments.

Statistical methods

A SigmaPlot software package (SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. The results, whenever applicable, were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test if significant differences ($P<0·05$) were observed.

Results

Effects of biochanin A on 7,12-dimethylbenz[a]anthracene–DNA adduct formation

To analyse the effects of the isoflavone on DMBA metabolism, [^{3}H]DMBA was added to MCF-7 cells in the presence or absence of isoflavone and the amount of [^{3}H]DMBA–DNA adduct was measured by scintillation counting. Biochanin A co-treated cultures had less [^{3}H]DMBA–DNA adduct formation/μg DNA than the DMBA control at all three concentrations (1·0, 5·0, 10·0 μM) tested (Fig. 1).

Effect of biochanin A on ethoxyresorufin-O-deethylase activity in MCF-7 cells

EROD activity was increased by more than 2-fold in DMBA-treated cultures compared with control cultures as illustrated in Fig. 2. Biochanin A co-treatment (1·0, 2·5 and 5·0 μM) significantly ($P<0·05$) suppressed this induction. The activities were normalized by cell viability results. The cell viability test did not reveal any significant differences among treatments in 24 h of incubation.

Kinetic analysis of the inhibition of cytochrome P450 1 enzymes by biochanin A

Because EROD encompassed both CYP1A1 and -1B1 enzyme activities, we further determined the specific inhibition using recombinant CYP1 enzymes. Lineweaver–Burk and inset plots suggested that biochanin A was a mixed-type inhibitor of CYP1A1 (Fig. 3(A)) and a competitive inhibitor of CYP1B1 (Fig. 3(B)). Based on the corresponding inhibition constant (K_i) values 4·00 and 0·59 μmol/l, biochanin A appeared to be selectively inhibiting CYP1B1 activities.

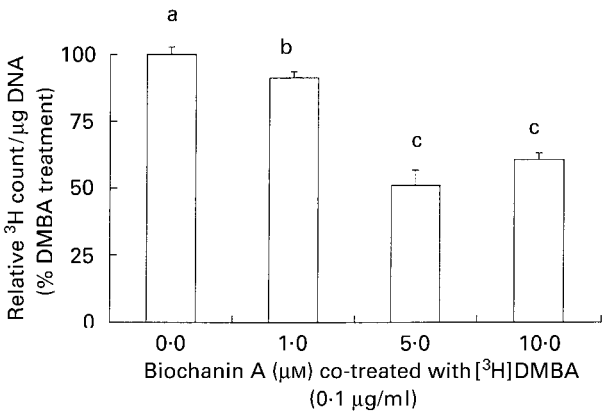

Fig. 1. Effect of biochanin A on 7,12-dimethybenz[a]anthracene (DMBA)–DNA adduct formation. MCF-7 cells were cultured in six-well plates and treated with [^{3}H]DMBA in 0·1 μg dimethylsuf-oxide/ml and biochanin A. After 24 h of treatment, genomic DNA was isolated and the DMBA–DNA lesions were determined by scintillation counting. The counts represented the amount of the DMBA metabolites conjugated to the DNA samples. For details of procedures, see p. 88. Values are means for three determinations with their standard errors shown by vertical bars. ^{a,b,c}Mean values with unlike superscript letters were significantly different ($P<0·05$).

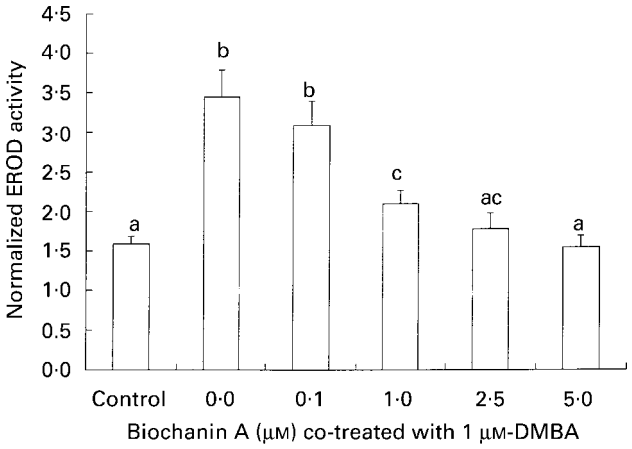

Fig. 2. Effect of biochanin A on ethoxyresorufin-*O*-deethylase (EROD) activities in cells. MCF-7 cells were seeded in ninety-six-well culture plates, and 1 μM-7,12-dimethylbenz[a]anthracene (DMBA) and various concentrations of biochanin A were co-administered. After 24 h treatment, cells were assayed for EROD activity. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assays were used to correct the differences in cell numbers. For details of procedures, see p. 88. Values are normalized means for six determinations with standard errors shown by vertical bars. [a,b,c]Mean values with unlike superscript letters were significantly different ($P<0{\cdot}05$).

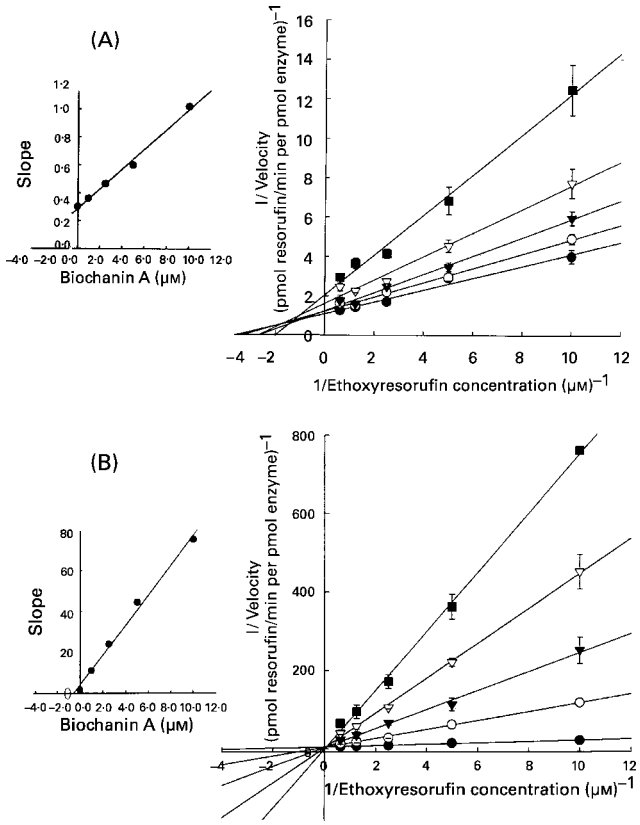

Fig. 3. Lineweaver–Burk plot of cytochrome P450 (CYP) 1 enzyme inhibition. Ethoxyresorufin-*O*-deethylase assay was performed on recombinant human CYP1A1 (A) and CYP1B1 (B) at the indicated concentrations of biochanin A and 100–1600 nM-ethoxyresorufin as substrate as described on p. 88. Lineweaver–Burk plots were generated by linear regression of the reciprocal data. •, 0·0 μM; ○, 1·0 μM; ▼, 2·5 μM; ▽, 5·0 μM; ■, 10·0 μM. The inset figure, which is a replot of the slopes from the Lineweaver–Burk plot, indicates the derivation of the inhibition constant (*K*i). For details of procedures, see p. 89. Values are means for three determinations with their standard errors shown by vertical bars.

Xenobiotic response element-driven luciferase activities

XRE reporter assay was subsequently measured to reveal AHR-mediated transcription. DMBA treatment increased the luciferase activity by 5-fold. The additions of 5·0 and 10·0 μM-biochanin A significantly reduced DMBA-induced luciferase activities (Fig. 4(A)). By itself, biochanin A (1·0 μM) was a weak inducer of the XRE-dependent transactivation (Fig. 4(B)).

Biochanin A inhibits 7,12-dimethylbenz[a]anthracene-induced cytochrome P450 1A1 and 1B1 mRNA expressions

The gel image of reverse transcription–PCR was analysed by densitometry. The optical densities of the CYP1A1 and -1B1 reverse transcription–PCR fragments normalized by that of β-actin are shown in Fig. 5(A and B). The corrected optical density indicated that DMBA increased CYP1A1 and CYP1B1 mRNA expressions by 5·0- (Fig. 5(B)) and 2·5- (Fig. 5(C)) fold, respectively. Biochanin A (5·0 μM) could abate ($P<0{\cdot}05$) the increases of both CYP1A1 and -1B1 mRNA abundance.

Discussion

In the present cell culture study, we showed that biochanin A reduced DMBA–DNA adduct formation and could be a chemopreventive agent. The interference of CYP1 enzyme activities was the major contributing factor to its mechanism. Biochanin A decreased CYP1A1 and -1B1 mRNA expressions induced by DMBA. Since a similar trend was seen in the XRE-driven luciferase activity, the scaling back of the RNA abundance appeared to be controlled by AHR transactivation. At the enzyme level, kinetic studies indicated that biochanin A was a strong inhibitor of CYP1B1 and -1A1.

CYP1A1 and -1B1 enzymes biotransform DMBA, and its metabolites may attack biological macromolecules (Gonzalez & Gelboin, 1994). AHR is an important modulator in the transcriptional control of *cyp1a1* and *cyp1b1* genes. MacDonald *et al.* (2001) suggest that phytochemicals with similar planar structure as AHR ligands may also act as inducers for *cyp1* transcription. That might partly explain the weak induction of biochanin A (1·0 μM) on XRE-driven transcriptional activity in the current study. Nonetheless, a ligand-independent XRE transactivation pathway has also been described in rat hepatoma cells (Backlund *et al.* 1997).

Many phyto-compounds have demonstrated their ability to modulate enzyme activity or gene expression of CYP1, or both. Galangin (Ciolino *et al.* 1999*a*), curcumin (Ciolino *et al.* 1998), quercetin (Ciolino *et al.* 1999*b*) and baicalein (Chan *et al.* 2002) display counteracting and/or potentiating activities toward XRE-dependent transactivation. They are able to stimulate CYP1A1 mRNA

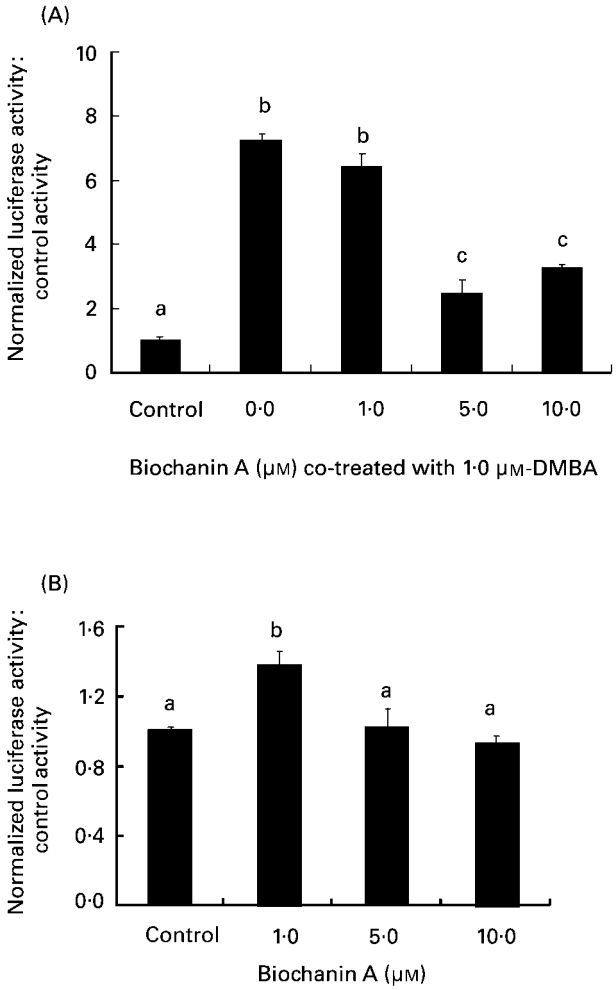

Fig. 4. Effect of biochanin A on xenobiotic response element (XRE)-driven luciferase activities. MCF-7 cells were transiently transfected with a luciferase reporter gene containing XRE and a renilla luciferase control plasmid. Biochanin A was co-administered with (A) or without (B) 1 μM-1,12-dimethylbenz[a]anthracene (DMBA) for 24 h. The arbituary light units of firefly luciferase were normalized by that of renilla luciferase, and their activities were measured by a dual-luciferase assay kit (Promega, Madison, WI, USA). For details of procedures, see p. 88. Values are means for three determinations with their standard errors shown by vertical bars. [a,b,c]Mean values with unlike superscript letters were significantly different ($P<0·05$).

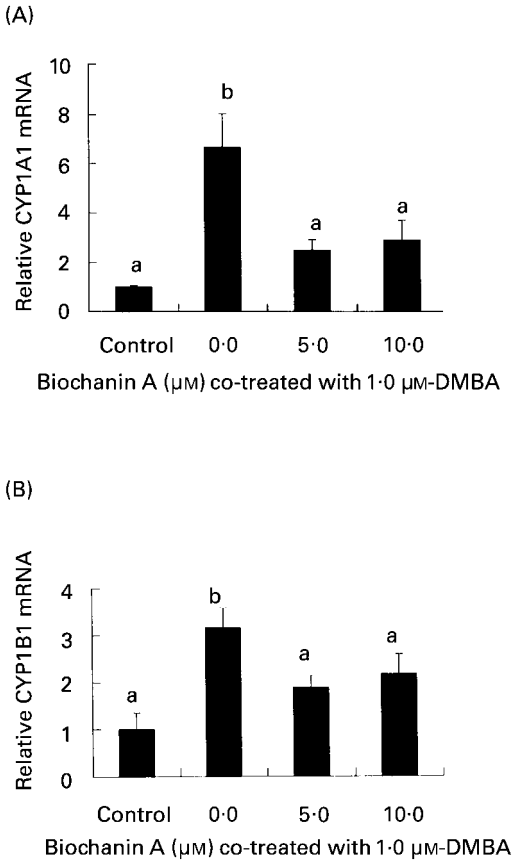

Fig. 5. Effect of biochanin A on cytochrome P450 (CYP) 1A1 and -1B1 mRNA expression. MCF-7 cells were treated with 1 μM-7,12-dimethylbenz[a]anthracene (DMBA) and biochanin A, and cultured for 24 h. Messenger RNA expressions of CYP1A1 (A) and CYP1B1 (B) were quantified by reverse transcription–polymerase chain reaction described in p. 88. Values are the results for the optical density of the targeted polymerase chain reaction fragments normalized by β-actin. For details of procedures, see p. 88. Values are means for three determinations with their standard errors shown by vertical bars. [a,b]Mean values with unlike superscript letters were significantly different ($P<0·05$).

expression by themselves, but they also antagonize the same expression induced by DMBA. Similar effects at the enzyme level are also observed. Kaempferol (Ciolino *et al.* 1999*b*) and resveratrol (Ciolino & Yeh, 1999), on the other hand, reduce PAH-induced CYP1A1 mRNA, but they are not AHR ligands. Various flavonoids might exhibit differential stimulatory or inhibitory effects on the CYP protein at the enzyme and expression levels. In the current study, we demonstrated that biochanin A was an inhibitor of EROD and a modulator of CYP1A1 and -1B1 expressions.

The expression or enzyme activity of CYP1 could be affected by biochanin A or its metabolites in culture. In T47D and ZR-75-1 breast-cell cultures, Peterson *et al.* (1998) have shown that biochanin A can be metabolized to biochanin A sulfate, genistein, genistein sulfate, and the hydroxylated and methylated metabolites of biochanin A and genistein. The present study indicated that biochanin A could lower the DMBA-induced CYP1 mRNA expressions.

Previous studies have demonstrated the anti-mutagenic properties of biochanin A. By using the bacterial strain *Salmonella typhimurium*, Francis *et al.* have found that the mutagenicities of aflatoxin B1 (1989*a*) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1989*b*) are inhibited by biochanin A. As compared with the present study, the isoflavone also affects the metabolism of PAH. Hamster embryo cells treated with biochanin A have a decreased transforming rate of benzy[a]pyrene to benzy[a]pyrene-9,10-diol and benzy[a]pyrene-7,8-diol, and the benzy[a]pyrene–DNA adduct formation is less than that in the control cultures (Chae *et al.* 1991, 1992). These observations are in accordance with our current findings.

In summary, the present study illustrated that the isoflavone biochanin A isolated from red clover could

reduce genotoxicity caused by PAH, and the mechanism was related to the suppression of CYP1A1 and -1B1 activities.

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