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A potential protective mechanism of soya isoflavones against 7,12-dimethylbenz[a]anthracene tumour initiation

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Epidemiological studies indicate that Asian women have a lower breast cancer incidence compared with their counterparts in the West, and the difference has been related to soya consumption. Animal studies have suggested that soya may prevent dimethylbenz[a]anthracene (DMBA)-induced carcinogenesis in the breast. In the present study a cell culture model was developed to address the effect of soya isoflavones on the DMBA-induced DNA damage. DMBA is metabolized into a DNA-attacking moiety by two phase I cytochrome P450 (CYP) enzymes CYP1A1 and CYP1B1. DNA mutation caused by this genotoxic agent is a crucial step in cancer initiation. Substances that interfere with the CYP1 enzyme activities can affect the initiation. In the present study, genistein was found to be an effective inhibitor of recombinant human CYP1A1 and CYP1B1 with K_i of 15·35 and 0·68 μmol/l. The other soya isoflavone daidzein, on the other hand, did not demonstrate any significant inhibition of the enzyme activities. At the transcriptional level, DMBA induced the CYP1 enzyme expressions by stimulating the xenobiotic response element (XRE)-dependent transactivation pathway. When genistein (25 μmol/l) was co-administered with DMBA, the XRE-Luc activity the CYP1 mRNA abundances were significantly suppressed. The present study illustrated that the soya isoflavone genistein, but not daidzein, protected against DMBA genotoxicity.

Genistein: Isoflavones: Phase I cytochrome P450 enzymes: Dimethylbenz(a)anthracene lesions

Polycyclic aromatic hydrocarbons (PAH) are commonly found in our environment, and they can be isolated from diesel exhaust, barbequed meat, tobacco smoke, overheated cooking oil, etc (International Agency for Research on Cancer, 1983; Environmental Protection Agency, 1990). PAH are metabolized and transformed into DNA-attacking electrophiles in the body. The significance of these environmental toxicants in breast cancer can be inferred from the increased presence of PAH–DNA adducts in human breast tumours (Li *et al.* 1996).

Aryl hydrocarbon receptor (AHR) is a mediator in the transformation of procarcinogens to genotoxic moieties. After binding to a PAH, the cytosolic AHR translocates to the nucleus and dimerizes with an AHR nuclear translocator. The dimerization initiates transcriptions of a gene containing xenobiotic responsive elements (XRE) in its promoter region (Kronenberg *et al.* 2000). Cytochrome P450 (CYP) 1A1 and CYP1B1 enzymes, which are responsible for the biotransformation of PAH, are downstream genes of AHR transactivation (Dertinger *et al.* 2000; Safe, 2001). The importance of AHR and CYP1B1 enzyme in PAH-induced carcinogenesis is implicated in two gene-knockout mouse results; benzo[a]pyrene (BaP) cannot induce cancer in AHR null mice (Shimizu *et al.* 2000), and lower cancer incidence was observed in 7,12-dimethylbenz[a]anthracene (DMBA)-treated CYP1B1 knockout mice (Buters *et al.* 1999).

The significance of CYP1 family enzymes in human breast cancer is not clear. Both tumour and normal tissues of the breast express CYP1A1 and CYP1B1 (Spink *et al.* 1998*a*,*b*; Iscan *et al.* 2001). The inhibition of CYP1 enzymes appears to be beneficial in the prevention of DMBA–DNA adduct formation *in vivo* and *in vitro* (MacDonald *et al.* 2001; Kleiner *et al.* 2002). Polymorphisms with higher activity of CYP1A1 appear to be a risk factor for breast cancer in African-Americans (Taioli, 1999); so are the polymorphisms of CYP1B1 in Asian women (Zheng *et al.* 2000).

Breast cancer is one of the most common cancers in women. Asian countries have lower breast cancer incidences than the West; however, no difference in breast cancer incidence is found between Asian descendents and other women in America (Ziegler *et al.* 1993). These results suggest that the environment may play a part in

Abbreviations: AHR, aryl hydrocarbon receptor; BaP, benzo(a)pyrene; CYP, cytochrome P450; DMBA, 7,12-dimethylbenz(a)anthracene; ER, oestrogen receptor; EROD, ethoxyresorufin-*O*-deethylase; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; XRE, xenobiotic response element.

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the aetiology of breast cancer, and soya consumption has been one of the major leads of investigation. Soya protein (Hakkak *et al.* 2000), soya extract (Gallo *et al.* 2001) and the soya isoflavone genistein (Lamartiniere *et al.* 1995*a*,*b*; Fritz *et al.* 1998, Hilakivi-Clarke *et al.* 1999) reduce DMBA-induced mammary tumours and mammary gland CYP1 expression (Rowlands *et al.* 2001) in rats. These data suggest that soya phytochemicals or soya protein could be the active chemopreventive ingredient. However, negative results are also observed for the soya effect on DMBA- (Appelt & Reicks, 1999) and N-methyl-N-nitrosourea (NMU)- (Cohen *et al.* 2000) induced mammary carcinogenesis.

The interaction of soya isoflavones and oestrogen receptor (ER) has been a major focus of soya's protective effect on breast cancer. Studies have shown that MCF-7 cells proliferate when exposed to genistein at low micromolar concentrations (Wang & Kurzer, 1997; Breinholt & Larsen, 1998; Le Bail *et al.* 1998; Shao *et al.* 1998) and experience cell death at higher concentrations (Peterson & Barnes, 1991; So *et al.* 1997). These observations have been interpreted as the antagonistic property of genistein to ER in the presence of oestrogen. However, our laboratory (Leung & Wang, 2000; Po *et al.* 2002) has demonstrated that the genistein-induced death of MCF-7 cells is not related to the ER antagonistic effect.

Many animal studies have illustrated the protective effect of soya protein or isoflavones against DMBA-induced mammary carcinogenesis, and the mechanism is yet unclear. MCF-7 cells have similar expressions of AHR, CYP1A1 and CYP1B1 to the non-tumour-derived breast epithelial MCF-10A cells (Spink *et al.* 1998*a*,*b*), and MCF-7 cells have the advantages of simpler subculturing conditions and shorter doubling time than MCF-10 cells. With the assumption that XRE transactivation played a pivotal role in PAH genotoxicity, MCF-7 cells are useful in identifying chemopreventive compounds as suggested by Smith *et al.* (2001). By using this cell culture system, the CYP1 enzyme pathways were investigated at the level ranging from dietary to supplemental intake. It was postulated that genistein and daidzein were modulators of CYP1 enzymes at the transcriptional as well as the enzyme level.

Materials and methods

Chemicals

Genistein, daidzein, and DMBA were obtained from Sigma-Aldrich, Milwaukee, WA, USA. Ethoxyresorufin and DMBA were purchased from Sigma Chemicals (St Louis, MO, USA). The carrier solvent dimethyl sulfoxide was also obtained from Sigma Chemicals. All other chemicals, if not stated, were acquired from Sigma Chemicals.

Cell culture

MCF-7 cells (gift from Dr V. C. Jordan) were cultured in RPMI–1640 phenol red-free media (Sigma Chemicals) and 10% (w/v) fetal bovine serum (Invitrogen Life Technologies, Rockville, MD, USA) at 37°C and 5% (v/v) CO_2. Sub-confluent cell cultures were treated with DMBA and various concentrations of genistein or daidzein.

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

The assay method was performed as previously described (Ciolino & Yeh, 1999). In brief, MCF-7 cells in ninety-six-well plates were treated with 1 μM-DMBA and various concentrations of genistein or daidzein. The medium was then removed and the cells were washed twice by 100 μl PBS. Ethoxyresorufin-*O*-deethylase (EROD) activities, which are indicative of CYP1A1 and CYP1B1, were then carried out. To each well was added 50 μl of 5 μM-ethoxyresorufin in PBS with 1·5 mM-salicyclamide, which was then 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.

Enzyme inhibition assays

Recombinant CYP1A1 and CYP1B1 expressed in baculovirus-infected insect microsomes (Supersomes®) were purchased from Gentest Corp., Woburn, MA, USA. Protein (2 pmol) was incubated in 100 μl PBS, pH 7·2 with 400 nM-ethoxyresorufin and genistein or daidzein 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 cell viability

Cell viability was assessed by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide straining as described by Mosmann (1983). Briefly, MCF-7 cells were plated in ninety-six-well plates at 10^4 cells per well, and 1 μM-DMBA and various concentrations of genistein or daidzein were administered for 24 h. At the end of the treatment, 50 μl of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg/ml) was added and the cells were incubated at 37°C for 4 h. Cell viability was determined by the absorbance at 600 nm.

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

This assay was performed as previously described (Ciolino & Yeh, 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 [^{3}H]DMBA (0·1 μg/ml; Amersham, Arlington Heights, IL, USA) was administered with or without genistein or daidzein. 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-tri(hydroxymethyl)-aminomethane-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 the incubation. The nuclei were then lysed by 400 μl lysis buffer B (1 % (w/v) SDS in 0·5 M-tri(hydroxymethyl)-aminomethane, 20 mM-EDTA and 10 mM-NaCl, pH 9), followed by the treatment of 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 that attained a 260 nm:280 nm ratio of $>1{\cdot}9$ were used for scintillation counting.

Xenobiotic response element–luciferase gene reporter assay

Construction of xenobiotic response element-activated luciferase reporter gene. A fragment with five XRE elements 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/well in twenty-four-well plates. After 24 h, the cells were transiently transfected with 4·0 μg of the XRE reporter plasmid and 1·0 μg of renilla luciferase control vector pRL (Promega, Madison, WI, USA) in LipofectAmine (Invitrogen Life Technologies). After 16 h, the medium was removed and the cells were treated with 1 μM-DMBA and various concentrations of genistein or daidzein 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. The XRE transactivation activities represented by firefly luciferase light units were then normalized with that of renilla luciferase.

Semi-quantitative reverse transcription–polymerase chain reaction assay

A reverse transcription–PCR assay was used to quantify mRNA level. Total RNA was isolated from cells grown in six-well Costar plates in triplicates 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 formerly (Dohr *et al.* 1995) and a Perkin Elmer Thermocycler (GeneAmp PCR System 2400, Norwalk, CT, USA) were utilized to amplify the target cDNA separately after the first strand reaction. All PCR reactions consisted of dNTP (0·2 mmol/l), 2 μl cDNA, primers A and B (both 0·2 μmol/l), 1 × PCR buffer and 1 U Taq polymerase. The conditions were 94°C for 45 s, 65°C for 45 s, 72°C for 1 min, and a final extension period of 7 min at 72°C. The amplification cycles were 25 for CYP1A1, 23 for CYP1B1, and 19 for β-actin. The PCR products were separated on 1·8 % (w/v) agarose 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 Prism® 3·0 software package (GraphPad Software, Inc., San Diego, CA, 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 ($P<0{\cdot}05$) were observed. In order to compare the cell cytotoxicity between DMBA-treated samples and those samples treated with DMBA plus genistein or daidzein, *t* tests were also performed.

Results

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

To analyse the effects of soya isoflavones on DMBA metabolism, ^{3}H-labelled DMBA was added to MCF-7 cells in the presence or absence of isoflavones and the amount of [^{3}H]DMBA–DNA adduct was measured by scintillation counting. Genistein treatment resulted in a concentration-dependent reduction of [^{3}H]DMBA–DNA adduct formation (Fig. 1 (A)). However, daidzein did not reduce the adduct formation significantly until a pharmacological concentration of 25 μM was reached and the inhibition was minimal (Fig. 1 (B)).

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

CYP1A1 and CYP1B1 activities were measured as EROD activity in intact cells. DMBA caused approximately a 2-fold increase in EROD activity compared with controls (data not shown). Genistein suppressed this induction in a concentration-dependent manner, with IC_{50} of approximately 12 μM (Fig. 2 (A)). Nevertheless, the suppressive effect of daidzein was far less effective. Only <30 % of DMBA-induced EROD activity was inhibited by 50 μM-daidzein (Fig. 2 (C)). The data indicated that genistein was effective in suppressing DMBA-induced EROD activity. Treatment with the two isoflavones alone only had a slight inhibition on EROD activity in MCF-7 cells (Figs. 2 (B) and 2 (D)).

Kinetic analysis of the inhibition of cytochrome P450 1A1 and 1B1 enzymes by genistein

Because both DMBA–DNA adducts and enzyme activities were decreased in cultures treated with genistein but not daidzein, the inhibition of CYP1A1 and CYP1B1 catalytic

activities by genistein was further characterized. Since daidzein did not show a significant contribution to the adduct formation and enzyme inhibition, this compound was not pursued any further. Enzyme kinetic experiments using recombinant human CYP1A1 and CYP1B1 enzymes were carried out. The Lineweaver–Burk plots and replots showed that genistein exhibited mixed-type inhibition on CYP1A1 enzyme (Fig. 3 (A)) and competitively inhibited CYP1B1 enzyme (Fig. 3 (B)). Based on the corresponding K_i values of 15·35 and 0·68 μmol/l, genistein appeared to be an effective inhibitor on both CYP1A1 and CYP1B1 activities at physiological concentrations.

Xenobiotic response element-driven luciferase activities

MCF-7 cells were transfected with an XRE reporter construct and luciferase activity was subsequently measured to reveal AHR-mediated transcription. DMBA induced luciferase activity by more than 5-fold. The addition of 10 and 25 μM-genistein produced a significant reduction in DMBA-induced luciferase activity (Fig. 4 (A)), while daidzein had no effect at this level (data not shown). By itself, genistein was a weak inducer of the XRE-dependent transactivation (Fig. 4 (B)).

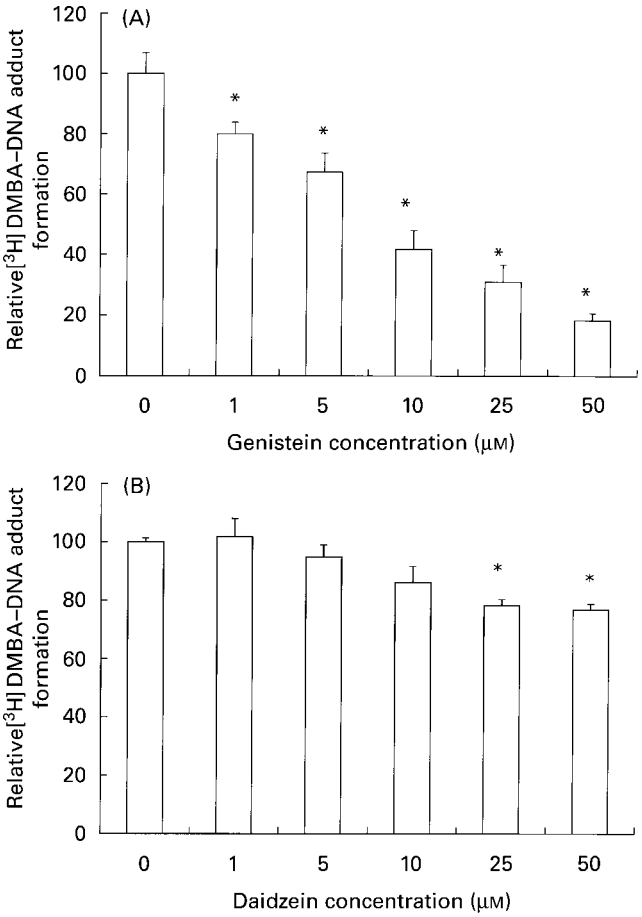

Fig. 1. Effects of isoflavones on 7,12-dimethylbenz[a]anthracene (DMBA)–DNA adduct formation in MCF-7 cells. MCF-7 cells were cultured in six-well plates and treated with ^{3}H-labelled DMBA (0·1 μg/ml) and co-administered with soya isoflavones at various concentrations; genistein (A) or daidzein (B). After 16 h of treatment, genomic DNA was isolated and the DMBA–DNA lesions were determined by scintillation counting. Values are means with their standard errors (*n* 3). *Mean values were significantly lower than that of the control (0 μM-genistein or -daidzein) ($P<0·05$).

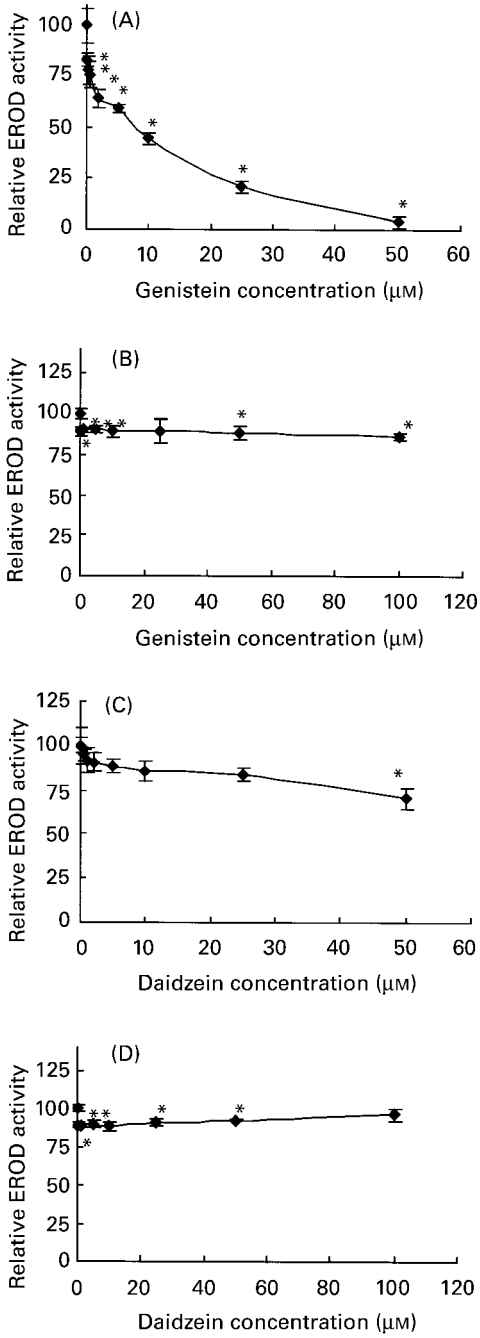

Fig. 2. 7,12-Dimethylbenz[a]anthracene (DMBA)-induced ethoxyresorufin-*O*-deethylase (EROD) activities in MCF-7 cells treated with soya isoflavones. MCF-7 cells were seeded in ninety-six-well culture plates and treated with (A and C) or without (B and D) 1 μM-DMBA and various concentrations of genistein (A and B) and daidzein (C and D). After 24 h of treatment, cells were assayed for EROD activity as described on p. 458. Values are means with their standard errors (*n* 6). *Mean values were significantly different from that of the control (0 μM-genistein or -daidzein) ($P<0·05$).

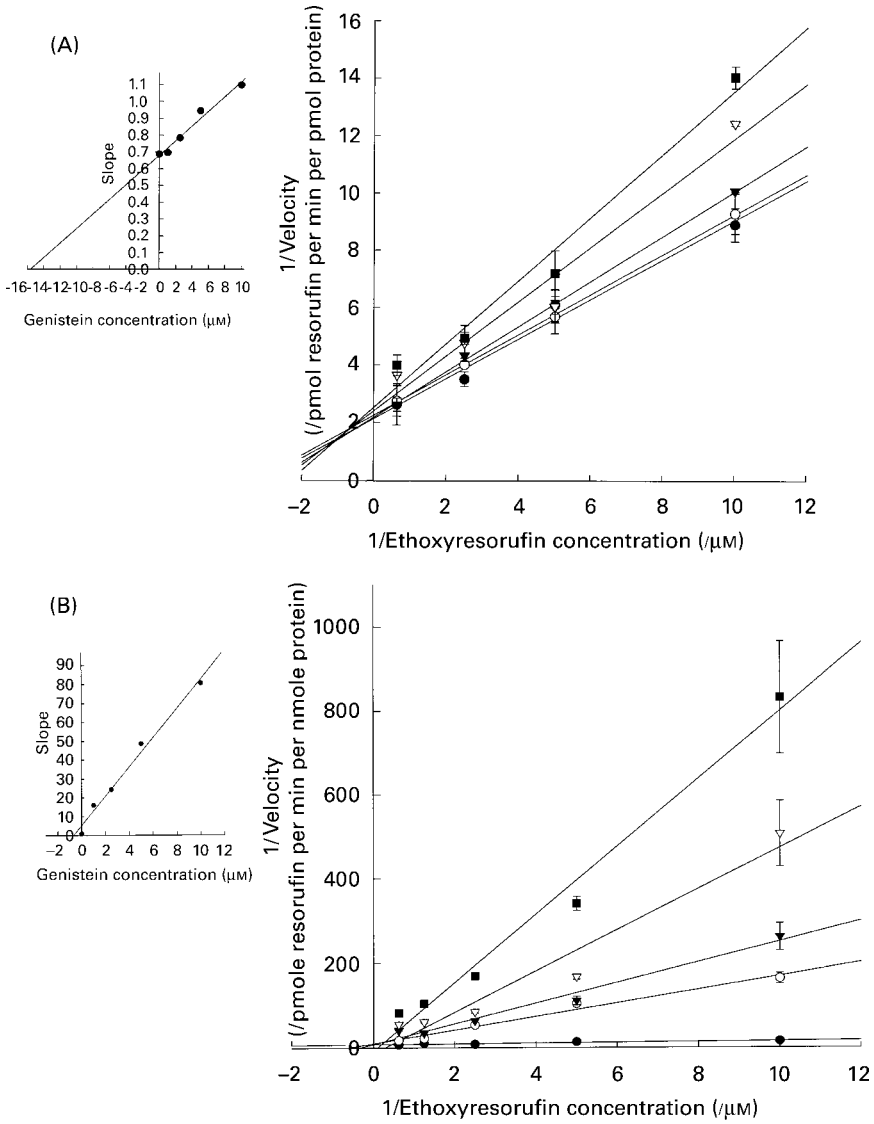

Fig. 3. Lineweaver–Burk plots of genistein on cytochrome P450 (CYP) enzymes CYP1A1 and CYP1B1 inhibition. Ethoxyresorufin-*O*-deethylase assay was performed with human recombinant CYP1A1 (A) and CYP1B1 (B) at various concentration of genistein (●), 0 μM; ○, 1·0 μM; ▼, 2·5 μM; ▽, 5·0 μM; ■, 10 μM) and 100–1600 nM-ethoxyresorufin as described on p. 458. Right, Lineweaver–Burk plots were generated by linear regression of the reciprocal data. Left, replot of the slopes from the Lineweaver–Burk plot, with derivation of K_i. Values shown are means with their standard errors (*n* 3).

Inhibition of 7,12-dimethylbenz[a]anthracene-induced cytochrome P450 1A1 and 1B1 mRNA levels by isoflavones

Because the XRE-dependent transactivation stimulated by DMBA administration was not affected until 10 μmol/l, CYP1A1 and 1B1 gene expressions were also examined to confirm the response. As estimated by the optical density of the images (Fig. 5 (A)), DMBA induced CYP1A1 and CYP1B1 mRNA expressions by 6·6- and 3·1-fold above basal levels, respectively. The increases were abated with genistein co-treatment at the concentration of 25 μmol/l (Figs. 5 (B) and (C)) for both CYP1A1 and CYP1B1. Genistein concentrations at 5 or 10 μmol/l did not significantly ($P<0·05$) decrease the mRNA abundance. The observations were consistent with the XRE data.

Discussion

Previous animal studies (Giri & Lu, 1995; Fritz *et al.* 1998) have demonstrated that isoflavones inhibit DMBA–DNA adduct formation, and MCF-7 cells can be a viable model to study the underlying mechanisms (Upadhyaya & El-Bayoumy, 1998). Making use of this *in vitro* model, the present study showed that genistein reduced DMBA–DNA adduct formation and the chemopreventive mechanism may be attributed to its interruption of DMBA metabolism. Daidzein, on the other hand, was shown to be non-functional in this regard. Genistein could down regulate CYP1A1 and 1B1 mRNA expressions at 25 μmol/l through its influence on XRE-dependent transcriptional control. At the enzyme level, kinetic studies indicated that the K_i values of CYP1A1 and 1B1 were

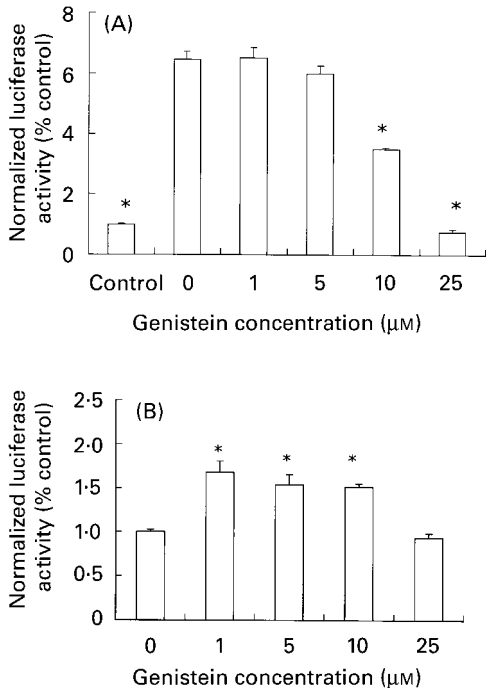

Fig. 4. Effects of genistein on 7,12-dimethylbenz[a]anthracene (DMBA)-induced xenobiotic response element (XRE)-driven luciferase activities. MCF-7 cells were transiently transfected with a luciferase reporter gene containing XRE and a renilla luciferase control plasmid. Genistein was co-administered with (A) or without (B) 1 μM-DMBA for 24 h. Values are means with their standard errors (n 3). *For (A), means were significantly ($P<0{\cdot}05$) lower than the cultures treated with DMBA only (0 μM-genistein). For (B), means were significantly higher than the control cultures (0 μM-genistein).

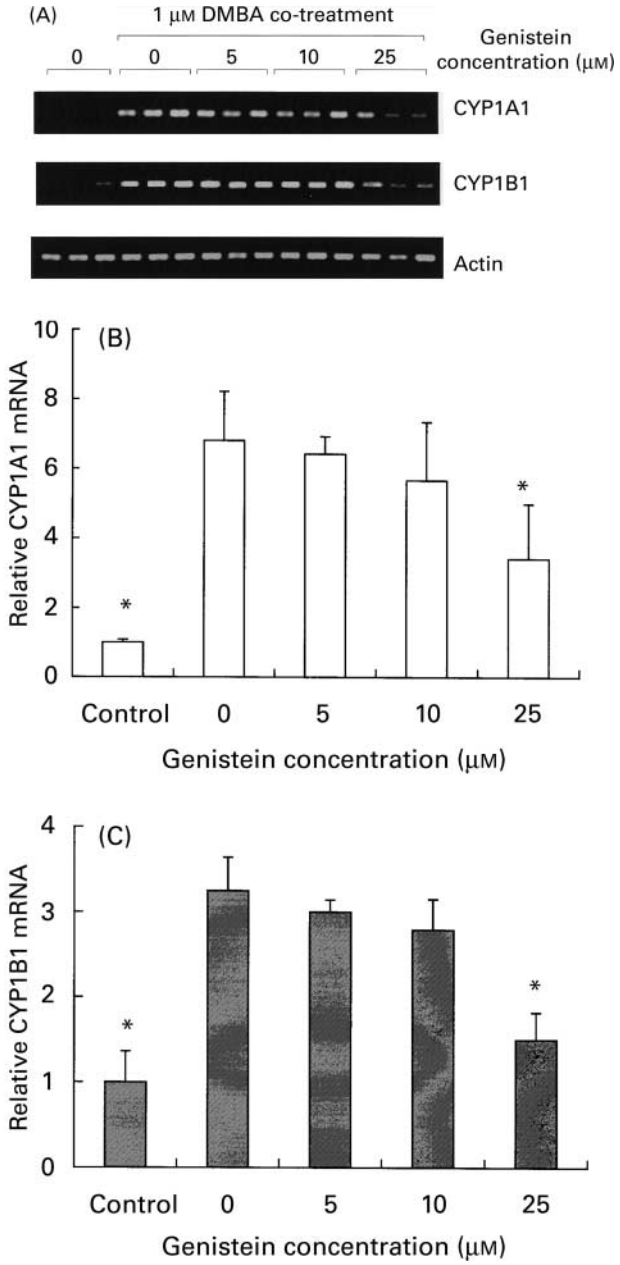

Fig. 5. Effects of genistein on 7,12-dimethylbenz[a]anthracene (DMBA)-induced cytochrome P450 (CYP) enzymes CYP1A1 and CYP1B1 mRNA expression. MCF-7 cells were treated with 1 μM-DMBA and genistein, and cultured for 24 h. mRNA expressions of CYP1A1 and CYP1B1 were quantified by reverse transcription–polymerase chain reaction (PCR) as described on p. 459. (A), gel image of the ethidium bromide-stained PCR fragments. (B and C), corresponding optical density results for co-administration with or without 1 μM-DMBA respectively. Values are means with their standard errors (n 3). *Mean values were significantly ($P<0{\cdot}05$) lower than the cultures treated with DMBA only (0 μM-genistein).

15·35 and 0·68 μmol/l, respectively. Considering that high soya consumption could bring about 0·5 μmol genistein/l in blood (Morton *et al.* 2002), the inhibition at the enzyme level appeared to be more significant than that at the expression level. Moreover, genistein appeared to preferentially inhibit CYP1B1 to CYP1A1 with the consideration of the different K_i values. In a previous study, the mammary CYP1B1 expression was shown to be higher than that of the liver (Horn *et al.* 2002). This might imply that the isoflavone could offer a stronger protection of PAH-induced carcinogenesis in the mammary gland than in the liver.

CYP1 enzymes biotransform DMBA, and its metabolites may attack biological macromolecules (Gonzalez & Gelboin, 1994). In the present study, it was demonstrated that genistein but not daidzein was an inhibitor of human CYP1 at the enzyme as well as at the transcriptional level. In contrast, Shertzer *et al.* (1999) have shown that both genistein and daidzein competitively inhibit BaP hydroxylation activities with IC_{50} of 140 and 325 μmol/l in a mouse hepatoma cell line. BaP–DNA adducts are significantly lower in cultures treated with 1 nM-2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 25 μM-genistein or -daidzein. Helsby *et al.* (1998) also performed a similar enzyme inhibition study on CYP1A in β-naphthoflavone-induced mouse liver microsomes, and the IC_{50} of genistein is in the millimolar range. The TCDD or β-naphthoflavone administered in these two studies may interfere with subsequent enzyme kinetic and BaP–DNA adduct assays, because the residual chemicals remaining in the microsomes can be significant. After all, the differences among those previous studies and the present study could be due to species, cell type, inducer and substrate variations.

The structure–inhibitory activity relationship has been described in xenobiotic-induced hepatic S9 fraction (Lee *et al.* 1994). The hydroxyl groups at the C4′ and C7 positions of the isoflavone molecules and the phenolic group at C5 are critical for the inhibitory action of EROD (Chae *et al.* 1992; Lee *et al.* 1994). As a result, daidzein that has hydroxyl groups at positions 4′ and 7 but lacking a C5 hydroxyl group may not be as active as genistein. The weak enzyme inhibitory effect of daidzein found in the present study was consistent with this structure–activity relationship.

Although AHR activation is a major pathway that controls the transcriptional activity of XRE-containing genes, an alternative AHR-independent mode of transactivation has also been documented (Backlund *et al.* 1997). In addition, MacDonald *et al.* (2001) suggest that phytochemicals with similar planar structure as AHR ligands may also act as inducers for CYP1 transcription. The weak induction of genistein on XRE-driven transcriptional activity in the present study may be dependent or independent of AHR activation.

Animal studies have elicited conflicting results on the cancer-protective effect of soya isoflavones. Although prepubertal administration of genistein could reduce breast cancer incidence in rats (Hilakivi-Clarke *et al.* 2000; Lamartiniere *et al.* 2002), soya given after weaning appears to be ineffective on DMBA-induced carcinogenesis (Appelt & Reicks, 1999). In contrast, Gallo *et al.* (2001) have shown that genistein does not protect DMBA-induced mammary tumour incidence or multiplicity but it reduces the percentage of poorly differentiated tumours. Contradictory to its cancer-protective implication, genistein has been demonstrated to increase DMBA-induced mammary tumours in ERα-intact mice (Day *et al.* 2001), and encourages the proliferation of MCF-7 tumours in athymic mice (Ju *et al.* 2001). In a recent study, daidzein and soya protein rather than genistein have been suggested to be the active ingredients in soya that reduce the multiplicity of DMBA-induced mammary tumours in rats (Constantinou *et al.* 2001). Nevertheless, both daidzein and genistein are effective in delaying the latency of mammary tumour development in a spontaneous carcinogenesis model, although the size and number of tumours are similar at the end of the experiment (Jin & MacDonald, 2002). These reports appear to be inconsistent regarding the chemopreventive effect of genistein, but the confounding results could be due to the phytochemical's differential actions on the initiation, promotion, and progression stages. The present study illustrated that genistein could be a chemopreventive agent targeting the tumour initiation phase.

The major soya isoflavone metabolite in women is in the glucuronide form, and the aglycone genistein only constitutes about 0·25 of total genistein present in plasma (Zhang *et al.* 2003). In a high soya-consuming country such as Japan, the average plasma concentration of total genistein is around 0·5 μmol/l in women (Morton *et al.* 2002). Although the lowest effective concentration (i.e. 1 μmol aglycone genistein/l in the present study) may not be achievable purely through dietary intake, it is still possible to reach that concentration by supplementation (Izumi *et al.* 2000). In addition, the hormone-responsive tissues have a higher genistein concentration than that of serum (Chang *et al.* 2000).

In conclusion, the present study identified genistein as the active ingredient in soya that inhibits DMBA–DNA adduct formation. Because CYP1 gene expressions did not alter below 10 μmol/l, the inhibition at the enzyme activity level was the mechanism of action in the low concentration range.

Acknowledgements

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