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 Ki 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 and CYP1 mRNA abundances were significantly suppressed. The present study illustrated that the soya isoflavone genistein, but not daidzein, protected against DMBA genotoxicity.

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\(^a\) (Hakkak\ et al.\, 2000\) and 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 earlier.\(\)

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 \(\mu\)M-DMBA and various concentrations of genistein or daidzein. The medium was then removed and the cells were washed twice by 100 \(\mu\)l PBS. Ethoxyresorufin-O-deethylase (EROD) activities, which are indicative of CYP1A1 and CYP1B1, were then carried out. To each well was added 50 \(\mu\)l of 5 \(\mu\)M-ethoxyresorufin in PBS with 1.5 \(\mu\)M-salicyclamide, which was then incubated at 37°C for 15 min. The reaction was stopped by 50 \(\mu\)l 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 baculo-virus-infected insect microsomes (Supersomes\®) were purchased from Gentest Corp., Woburn, MA, USA. Protein (2 pmol) was incubated in 100 \(\mu\)l PBS, pH 7.2 with 400 nm-ethoxyresorufin and genistein or daidzein in different concentrations. The reaction was initiated by 500 \(\mu\)M-NADPH, and stopped by 100 \(\mu\)l 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 staining as described by Mosmann (1983). Briefly, MCF-7 cells were plated in ninety-six-well plates at 10\(^3\) cells per well, and 1 \(\mu\)M-DMBA and various concentrations of genistein or daidzein were administered for 24 h. At the end of the treatment, 50 \(\mu\)l 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 \(\times\) 10\(^3\) cells per well and allowed to attach for 24 h. Then [\(^{3}\)H]DMBA (0.1 \(\mu\)g/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%...
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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 >1.9 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 10^3 cells/well in twenty-four-well plates. After 24 h, the cells were transiently transfected with 40 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<0.05) 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, [3H]labelled DMBA was added to MCF-7 cells in the presence or absence of isoflavones and the amount of [3H]DMBA–DNA adduct was measured by scintillation counting. Genistein treatment resulted in a concentration-dependent reduction of [3H]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 $\text{mmol/l}$, 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 $\mu$M-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 $\mu$g/ml) and co-administered with soya isoflavones at various concentrations; genistein (A) or daidzein (B). After 16h 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 $\mu$M-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 $\mu$M-DMBA and various concentrations of genistein (A and B) and daidzein (C and D). After 24h 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 $\mu$M-genistein or -daidzein) ($P<0.05$).
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...
15·35 and 0·68 mol/l, respectively. Considering that high soya consumption could bring about 0·5 mol 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 Ki 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 IC₅₀ of 140 and 325 mol/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 μmol-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 IC₅₀ 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.

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References


