Genistein protects human mammary epithelial cells from benzo(a)pyrene-7,8-dihydriodiol-9,10-epoxide and 4-hydroxy-2-nonenal genotoxicity by modulating the glutathione/glutathione S-transferase system

Claudia Steiner, Wilbert H.M.Peters¹, Evan P.Gallagher², Pamela Magee³, Ian Rowland³ and Beatrice L.Pool-Zobel*

Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller University Jena, Dornburger Straße 25, 07743 Jena, Germany, ¹Department of Gastroenterology, St Radboud University Hospital, Nijmegen, The Netherlands, ²Department of Environmental and Occupational Health Sciences, University of Washington, 4225 Roosevelt Way NE, Suite 100, Seattle Washington, DC, USA and ³Northern Ireland Centre for Diet and Health, University of Ulster, Coleraine, Northern Ireland BT52 1SA

*To whom correspondence should be addressed. Tel: +49 0 3641 949670; Fax: +49 0 3641 949672; Email: blpob@uni-jena.de

Epidemiological studies have shown that ingestion of isoflavone-rich soy products is associated with a reduced risk for the development of breast cancer. In the present study, we investigated the hypothesis that genistein modulates the expression of glutathione S-transferases (GSTs) in human breast cells, thus conferring protection towards genotoxic carcinogens which are GST substrates. Our approach was to use human mammary cell lines MCF-10A and MCF-7 as models for non-neoplastic and neoplastic epithelial breast cells, respectively. MCF-10A cells expressed hGSTA1/2, hGSTA4-4, hGSTM1-1 and hGSTP1-1 proteins, but not hGSTM2-2. In contrast, MCF-7 cells only marginally expressed hGSTA1/2, hGSTA4-4 and hGSTM1-1. Concordant to the protein expression, the hGSTA4 and hGSTP1 mRNA expression was higher in the non-neoplastic cell line. Exposure to genistein significantly increased hGSTP1 mRNA (2.3-fold), hGSTP1-1 protein levels (3.1-fold), GST catalytic activity (4.7-fold) and intracellular glutathione concentrations (1.4-fold) in MCF-10A cells, whereas no effects were observed on GST expression or glutathione concentrations in MCF-7 cells. Preincubation of MCF-10A cells with genistein decreased the extent of DNA damage by 4-hydroxy-2-nonenal (150 μM) and benzo(a)pyrene-7,8-dihydriodiol-9,10-epoxide (50 μM), compounds readily detoxified by hGSTA4-4 and hGSTP1-1. In conclusion, genistein pretreatment protects non-neoplastic mammary cells from certain carcinogens that are detoxified by GSTs, suggesting that dietary-mediated induction of GSTs may be a mechanism contributing to prevention against genotoxic injury in the aetiology of breast cancer.

Introduction

Breast cancer is the most common cancer among women in countries with western style diet and the third most common cancer among women world-wide (1). Historically, the incidence breast cancer rates are substantially lower in China, Japan and other Asian countries than in the USA and Europe (2). Migrants from Asia to the USA typically acquire the breast cancer risk of their host nation by the second generation, which suggests a marked influence of environmental and life-style factors (3). The low prevalence of breast cancer in Asia is associated with the consumption of a diet abundant in soy (4). Specifically, it has been estimated that the approximate daily soy intake is 10–50 g in Asia, as compared with 1–3 g in the US and in Europe (5). Associated with this is an estimate that 20–100 mg isoflavones per day are ingested in Asia, whereas in Europe and in the USA daily intake is <1 mg (6–9).

Several observational epidemiological studies have examined the relationship between soy and breast cancer. Case-control studies examining the association between soy intake and breast cancer risk in Asian women (10,11) and Caucasian women (12) consistently suggest an inverse association for both pre- and post-menopausal breast cancer. It was also reported that the breast cancer preventive effects of dietary soy correlate with adolescent dietary exposure (11). There is substantial evidence that isoflavones contribute strongly to the chemoprotectant properties of soy products, and specifically with regards to inhibition of breast cancer development. Isoflavones constitute a group of phytoestrogens, i.e. naturally occurring non-steroidal plant compounds containing a diphenoquinone structure, that are able to exert estrogenic effects (13). These compounds can interact with human estrogen receptors (ER), inhibit their action, suppress cell growth and protect against the development of breast cancer (14). The isoflavone genistein has been a major focus of attention since it has a number of activities, which prevent the progression of initiated cells to tumours. These activities include induction of mammary gland differentiation in rodents (15), inhibition of cell proliferation by cell cycle modulation and induction of apoptosis (16,17), inhibition of angiogenesis (18) and tyrosine kinases (19). Interestingly, soy isoflavones such as genistein can also enhance DNA damage in different cell cultures at doses >1 μM (20–24). However, when mice or humans are exposed to dietary relevant or higher levels of genistein or isoflavones, genetic damage is not observed (22,25). Furthermore, studies in our laboratory have shown that doses of genistein up to 30 μM are not genotoxic in human mammary cell lines (C. Steiner, unpublished data). Thus, it appears that the genotoxic effects by genistein may be dose-specific, as well as dependent on the timing of exposure. Moreover, the genotoxic effects could be highly cell type-specific as well. For example, we have found that in human colonocytes, genistein induces strand breaks (100 μM

Abbreviations: AP-1, activator protein-1; ARE, antioxidant response element; BPDE, benzo(a)pyrene-7,8-dihydriodiol-9,10-epoxide; BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; ERE, estrogen responsive element; ERK1/2, extracellular regulated protein kinase 1/2; GSH, reduced glutathione; GST, glutathione S-transferase; 4-HNE, 4-hydroxy-2-nonenal; MAPK, mitogen-activated protein kinases; NQO1: NAD(P)H quinone oxidoreductase; PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species.
exposure for 15 min) (26) whereas in human prostate cells, no increase in DNA damage was observed (80 μM exposure for 1 h (27).

Blocking agents such as phytoestrogens have promise for overall cancer risk reduction (28) by reducing exposure to genotoxic metabolites at critical cell targets through the induction of detoxification genes (29–31). Of particular importance with respect to the detoxification of carcinogenic intermediates are the GSTs, a supergene family of cytosolic, mitochondrial and microsomal enzymes (32), most of which have the ability to conjugate electrophilic chemicals with glutathione (GSH) (33). The cytosolic transferases are represented by the classes alpha, mu, pi, sigma, theta, omega and zeta. In addition to the cytosolic forms, mitochondrial GSTs comprise the kappa class GST, whereas microsomal GSTs represent a distinct gene family of membrane-associated proteins that are active in eicosanoid and glutathione metabolism (32). Several GST isoforms are also of importance in protecting against oxidative stress by conjugating reactive quinones arising from catecholestrogens, reactive α, β-unsaturated aldehydes produced during lipid peroxidation, and also by reducing organic hydroperoxides via selenium-independent glutathione peroxidase activity (34,32,35). Although GSTs are constitutively expressed in human tissues, tissue- and cell-specific isoform expression shows marked variability, a factor which can greatly influence susceptibility to toxicity and disease (32).

A particularly important data gap is a lack of information regarding GST isoform expression in breast tissue. The most prevalent isoenzyme in extrahepatic tissues (36) and the major GST isoenzyme in human breast tissue (37) hGSTP1-1 is inducible by flavonoids and isothiocyanates (38,39), by the gut fermentation product butyrate (40,41), as well as by antiestrogens (37). In the present study, we characterized GST isoform expression in human mammary epithelial cells and tested the hypothesis that genistein induces GST isoforms and protects against the genotoxic injury of the mutagenic compounds 4-HNE and BPDE. We used MCF-7, a mammary carcinoma cell line frequently used in studies involving genistein and related chemoprotective compounds. We also used MCF-10A cells, since they are derived from non-malignant tissue and exhibit a stable karyotype appropriate for genotoxic and gene expression studies (42) targeting mechanisms of chemoprotection that occur prior to initiation.

### Materials and methods

**Chemicals**

Cell culture materials, including Dulbecco’s Modified Eagle Medium (DMEM), non-essential amino acids (MEM), penicillin/streptomycin, fetal calf serum (FCS) and trypsin were obtained from InVitrogen GmbH (Karlsruhe, Germany). Mammary Epithelial Growth Medium (MEGM) was provided by Promocell (Heidelberg, Germany), charcoal-stripped fetal calf serum (CCS) by Biological Industries (Israel) and trypsin/EDTA solution purchased from Biochrom AG (Berlin, Germany). Genistein was supplied by TCI Tokyo Kasei (Japan). Choleratoxin, DMSO, metaphosphoric acid, 3,3'-diiodobenzidine (DAB) and hydrogen peroxide were purchased from Sigma-Aldrich GmbH (Steinheim, Germany), TRizol® reagent, deoxyribonuclease I, and SuperScript™ First-Strand Synthesis System were purchased from InVitrogen GmbH (Karlsruhe, Germany). The QiAamp Mini Kit and Hot Star Taq DNA Polymerase were provided by Qiagen GmbH (Hilden, Germany). The RNaseasy Mini Kit was provided by Qiagen Ltd. (Crawley, UK). Rabbit anti-mouse Ig conjugated with horseradish peroxidase was purchased from DAKO (Hamburg, Germany), and goat anti-chicken IgY conjugated with horseradish peroxidase from CalBiotech (Santa Cruz, USA). Enhanced chemiluminescence (ECL)™ western blotting detection reagents were provided by Amersham Biosciences Europe GmbH (Freiburg, Germany). 1-chloro-2,4-dinitrobenzene (CDNB) and GST were obtained from Merck (Darmstadt, Germany). The Glutathione Assay-Kit and 4-hydroxy-2-nonenal (4-HNE) were purchased from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Benzotri/pyrene-7,8-dihydropyridol-9,10-epoxide (BPDE) was provided by Biochemical Institute for Environmental Carcinogens (Grosshansdorf, Germany).

**Cell culture experiments and exposures**

Two cell lines, MCF-10A and MCF-7, differing in the transformation stage were chosen for the experiments. The non-tumorigenic cell line MCF-10A derived from reduction mammaplasty tissue (43) was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in MEGM supplemented with 100 ng/ml of cholera toxin at 37 °C in humidified atmosphere (95% air, 5% CO2). The mammary tumour cell line MCF-7 (44) was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were maintained in DMEM supplemented with 10% (v/v) FCS, 1% (v/v) MEM and 1% (v/v) penicillin/streptomycin at 37 °C in a humidified incubator (95% air, 5% CO2). MCF-10A cells have only a marginal ERβ expression whereas MCF-7 cells show both ERα and ERβ expression (45). The cells were trypsinized with 1.5 ml of trypsin/EDTA and 10% trypsin, respectively, for up to 4 min and sub-cultured at a dilution of 1:4 with culture medium. The medium was changed every 3 days, and passages 7–30 were used for the experiments. Prior to experiments, cells were transferred to phenol-red free medium containing CCS in order to eliminate potential estrogenic compounds of the serum.

In chemoprotection experiments, the MCF-7 and MCF-10A cells were plated in 25 cm2 cell culture flasks and incubated with genistein at concentrations of 1–30 μM, with a final DMSO concentration of 0.1%. These concentrations were based on sub-toxic doses (IC50) determined previously for these cell lines (C. Steiner, C.E. Rüfer, S.E. Kulling, K. Wahaîla, B.L. Pool-Zobel, manuscript in preparation). After 48 h of treatment, the cells were trypsinized and washed in cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4·2H2O, 1.5 mM KH2PO4 and 0.5% BSA; pH 7.4). Cell number and cell viability were determined with trypan blue dye exclusion using a haemocytometer, and analysis of GST expression, GSH concentrations and DNA damage were conducted as described below.

### Comparative analysis of GST isoenzyme mRNA expression in the breast cancer cell lines by semi-quantitative RT–PCR

Total RNA was isolated using the TRizol method. Following DNA digestion, cDNA equivalent to 5 μg total RNA was prepared by first-strand synthesis using oligo(dT) primer. Semi-quantitative reverse transcriptase PCR was then used to characterize steady-state GST mRNA expression pattern for hGSTA1, hGSTA2, hGSTA4, hGSTM1, hGSTM2, hGSTP1 and hGSTT1 in the breast cell lines (sequences of the primers and their product sizes are listed in Table I). GST29 colon cells were used, as a positive control cell line, for GST genotyping and also for GST mRNA expression by RT–PCR. The PCR conditions were as follows: 10 ng total cDNA, 1 unit of Hot Star Taq DNA Polymerase, 20 pM of primers and 1.5 mM MgCl2. The cDNA was amplified by PCR, with 38 cycles. After primary denaturation at 95 °C for 15 min, the PCR cycles were run at 94 °C for 30 s for denaturation, 55 °C (A1, A2), 60 °C (M1, M2, P1) or 63 °C (A4, T1) for 30 s for annealing and 72 °C for 1 min for extension.

**Effect of genistein on quantitative expression of hGSTA4 and hGSTP1 mRNA levels**

After incubation with genistein (1–30 μM) for 8, 24 and 48 h, breast cells were trypsinized and washed twice with PBS/BSA followed by the RNA isolation using RNeasy Mini Kit according to the manufacturer’s protocol. RNA quantity and quality were assessed spectrophotometrically (260/280 absorbance ratios) and by gel electrophoresis. After ensuring RNA quality, 500 ng RNA were reverse transcribed using random hexamer primers. A region of hGSTA4 and hGSTP1 mRNA was amplified using the primers GSTA4-F (5'-GGATCTGGAGAATGCCTTATC-3') and GSTA4-R (5'-GTTCGTGACCCCTTTAAAATCTT-3') and GSTP1-F (5'-CTACATC- AAAGCCCTCTGCTCAT-3') and GSTP1-R (5'-CAG GATGTTAGGACAG- GGTCACAG-3'). Primers and probes for the endogenous reference β-actin, as a ready-to-use kit, were supplied by Applied Biosystems (UK). TaqMan® real-time PCR was performed in 96-well plates, following the guidelines of the manufacturer (Applied Biosystems, USA). All experiments were performed in triplicate and a validation experiment was performed to confirm the specificity of the target amplification, and also the efficiency of the reference amplification. hGSTA4 and hGSTP1 mRNA expression was then normalized to the endogenous reference β-actin using the comparative CT method (ΔΔCT = ΔCTcontrol – ΔCTtreatment) according to the
manufacturer’s guidelines. The effect of genistein on GST mRNA expression change was calculated according to the efficiency method (fold change = 2^ΔΔCt), where it is assumed that the primer efficiency is 100%.

Genotyping of cell lines for GSTM1, GSTT1 and GSTP1 status

Genomic DNA was extracted from 1 × 10^6 cells using the QiAamp DNA Mini Kit following the manufacturer’s protocol. Multiplexed PCR was used to detect hGSTm1 and hGSTt1 deletion polymorphisms (46,47), whereas the sequence polymorphism of hGSTP1 at codon 104 (A→G transition) was detected using the restriction fragment length polymorphism (RFLP)–PCR (38).

Analysis of GST proteins and catalytic activity, and intracellular GSH concentrations

Prior to cytosol preparation, the cells were resuspended in cold homogenization buffer (250 mM sucrose, 20 mM Tris–HCl, 1 mM DTT and 1 mM EDTA). Following centrifugation (16 000 × g, 10 min) the supernatant was collected and stored at −80°C until use. Western blot analyses of GST proteins were performed as described previously (48). For these experiments, defined amounts of cytosolic protein were used for these experiments. For these experiments, defined amounts of cytosolic protein were used (MCF-7: up to 120 μg for hGSTT1-1; MCF-10A: up to 60 μg for hGSTT1-1, A1/2, M1-1, M2-2, and up to 80 μg for A4-4; MCF-10A: 25 μg for hGSTT1-1, up to 120 μg for hGSTA1/2, M1-1, M2-2 and up to 80 μg for A4-4) to ensure a semi-quantitative analysis. We used monoclonal antibodies against human GST class alpha, mu and pi (49). Specifically, the alpha class GST antibodies recognize human hGSTA1-1, A1-2 and A2-2 whereas the mu class GST antibodies react against human hGSTM1a-1a, M1a-1b and M1b-1b (50) and probably against hGSTM2-2. However, because of lack of the appropriate hGSTM2-2 standard protein, the detection of hGSTM2-2 was not validated in the present study. GST protein expression was detected with DAB and hydrogen peroxide after incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins as secondary antibody. Detection of hGSTA4-4 was accomplished using a polyclonal chicken anti-hGSTA4 peptide antibody as previously described (51). GST protein levels were quantitated by densitometry using Fluor S reader (52). GST protein concentrations were quantitated by densitometry using Fluor S reader (52). GST protein concentrations were quantitated by densitometry using Fluor S reader (52).

Table I. Primer sequences for RT–PCR of GSTs

{% raw %}
<table>
<thead>
<tr>
<th>GST genes</th>
<th>Sequence</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>forward</td>
<td>5'-AGC CCA AGC TTC ACT ACT TCA ATG C-3'</td>
</tr>
<tr>
<td>A2</td>
<td>forward</td>
<td>5'-GAT AAG ACT AGG GTA CAA CTC TTC C-3'</td>
</tr>
<tr>
<td>A4</td>
<td>reverse</td>
<td>5'-CCC GAT GGA GTC CTT GAG ATG G-3'</td>
</tr>
<tr>
<td>M1</td>
<td>forward</td>
<td>5'-GAA CTC GAA AGG CTA CAA ACG C-3'</td>
</tr>
<tr>
<td>M2</td>
<td>reverse</td>
<td>5'-GTC GGT GAC CAA TAT ACG GTG G-3'</td>
</tr>
<tr>
<td>P1</td>
<td>forward</td>
<td>5'-ACC CCA GGG CTC TAT GAG AA-3'</td>
</tr>
<tr>
<td>T1</td>
<td>reverse</td>
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</tr>
<tr>
<td>β-actin</td>
<td>forward</td>
<td>5'-GGT GTC GAT GGA GTC CGT CAG A-3'</td>
</tr>
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Determination of genetic damage (Comet assay)

The effects of genistein on 4-HNE- and BPDE-induced DNA damage were measured using the Comet assay. Cells were treated for 48 h with genistein (1–30 μM) or vehicle control (0.1% DMSO). Following the treatments, the cells were harvested, washed with ice cold PBS and incubated with 4-HNE or BPDE at 37°C for 30 min and at concentrations previously established in our laboratory to induce genotoxicity that are specific concentrations for the cell lines (MCF-10A: 150 and 50 μM; MCF-7: 75 and 20 μM, respectively).

Results

Comparative expression of GST mRNA and proteins in MCF-10A and MCF-7 cells

As observed in Table II, both mammary cell lines examined expressed considerable amounts of GST isoenzymes based on mRNA and protein measurements. However, the dysplastic cell line MCF-10A lacked detectable levels of hGSTA1, hGSTA2 and hGSTM2 mRNA, and the GSTT1*B1*0 polymorphism was also present in the cell line (Figure 1A). Both MCF-10A, as well as, MCF-7 cells exhibited the heterozygous GSTP1*A*B genotype (Figure 1B). As observed in Table II, both cell lines expressed functional hGSTA1/2, hGSTA4-4 and hGSTM1-1 proteins. In contrast, the hGSTM2-2 protein was not detectable in either mammary cell lines. Additionally, tumourigenc MCF-7 cells did not express the hGSTP1-1 protein. The higher protein expression...
of the breast cancer risk factor related hGSTA4-4 and hGSTP1-1 in the dysplastic cell line MCF-10A correlated with corresponding mRNA expression. MCF-10A cells expressed 2.5-fold more hGSTA4 mRNA (17.2 ± 9.4 ng in MCF-10A versus 6.8 ± 1.5 ng in MCF-7) and 66 000-fold greater hGSTP1 mRNA than MCF-7 cells (9.9 ± 1.1 ng in MCF-10A versus 0.00015 ± 0.00011 ng in MCF-7, Table III).

The lack of the hGSTP1-1 protein expression and the marginal expression of the alpha and mu class GSTs in MCF-7, all of which conjugate CDNB, were reflected by 100-fold lower GST activity in MCF-7 cells relative to MCF-10A cells (Table III). In contrast, overall GSH concentrations did not differ among the two cell types (Table III). In general, the MCF-10A cells expressed more overall GST protein and GST subunit levels than MCF-7 cells, consistent with the enzymatic analysis.

Modulation of hGSTP1 and hGSTA4 mRNA and protein expression, GST activity and intracellular GSH level by genistein

As observed in Figure 2A, exposure of MCF-10A cells to 30 μM of genistein elicited a significant 4.7-fold increase in overall GST-CDNB activity relative to controls. This effect was accompanied by an increase in cellular GSH concentrations (Figure 2B). The increased GST-CDNB activities in MCF-10A cells appear to be due to a significant induction of hGSTP1-1 protein (3.1-fold from 851 ± 261 to 2633 ± 390 ng/10^6 cells at 30 μM; Figure 3). There was a significant correlation between the hGSTP1-1 protein concentrations and the overall GST-CDNB activity at the significant modulating concentration of 30 μM. The modulation of hGSTP1-1 protein expression correlated with genistein-mediated changes in mRNA expression. Genistein marginally enhanced hGSTP1 mRNA expression after 8 h (30 μM, 1.3-fold) and 24 h (10 and 30 μM, 1.3- and 1.2-fold, respectively) of exposure. A further enhancement of hGSTP1 mRNA expression was observed after 48 h incubation (1, 10, 30 μM for 2.3-, 1.8- and 1.7-fold, respectively, Figure 4A). Although exposure to genistein (1–30 μM) did not affect hGSTA4-4 protein expression in MCF-10A cells (Table IV), it did down-regulate hGSTA4 mRNA expression (Figure 4B).

In contrast, exposure to genistein did not alter GST-CDNB activities or GSH concentrations in MCF-7 cells (Figure 2A and B). While hGSTP1-1 protein was not detectable in MCF-7 cells, hGSTA4-4 protein expression was not affected.
by genistein (Table IV). But as shown in Figure 4B, there was a time dependent modulation of \( h\text{GSTA4} \) mRNA expression. After a 1.3-fold increase of \( h\text{GSTA4} \) mRNA by 30 \( \mu \text{M} \) of genistein following 8 h treatment, the mRNA expression was decreased by 40–60% after 24 h. Increased temporal exposure to genistein did not influence \( h\text{GSTA4} \) mRNA expression (Figure 4B).

**Chemoprotection of DNA damage by genistein**

Figure 5 demonstrates that the level of DNA damage in MCF-10A cells on exposure to 4-HNE and BPDE (13.5 ± 1.3% tail intensity for 150 \( \mu \text{M} \) 4-HNE; 49.3 ± 6.4% tail intensity for 50 \( \mu \text{M} \) BPDE) was suppressed by genistein. Genistein-mediated chemoprotection against 4-HNE was clearly evident at low genistein concentrations, whereas

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**Fig. 2.** (A) Effect of 48 h genistein treatment on GST catalytic activity in MCF-10A and MCF-7 cells. Results are means ± SD of a minimum of three experiments. Significant effects in genistein-induced GST activities were calculated by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test (**\( P < 0.01 \)). (B) Effect of 48 h genistein treatment on intracellular GSH levels in MCF-10A and MCF-7 cells. Results are expressed in percentage increase in cellular GSH. The corresponding GSH concentrations in control MCF-10A and MCF-7 cells were 5.2 ± 1.9 and 6.7 ± 2.8 nmol \( \cdot 10^{-6} \) cells, respectively. The results represent means ± SD of seven and four experiments for MCF-10A and MCF-7 cells, respectively. Significant differences of genistein treatments were calculated by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test (**\( P < 0.01 \)).
Genistein modulates GSTs

In the present study, we have demonstrated that genistein may act as a blocking agent in breast cancer via induction of GST. In this regard, an up-regulated detoxification system may facilitate the detoxification and/or elimination of ultimate carcinogens, thereby eliciting an inhibitory effect against the initiation of cancer and potentially reducing the risk of tumourigenesis (28). Support for this hypothesis on blocking agents in cancer chemoprevention is evidenced by several in vivo and in vitro studies (39,59–61). Our data also revealed different expression patterns of GST among the two cell lines, with higher overall GST levels in non-transformed MCF-10A cells. The extremely low expression of GST isoforms hGSTA4 and hGSTP1 in MCF-7 cancer cells is noteworthy. The lack of detectable hGSTP1-1 protein in the presence of low amounts of hGSTP1 mRNA may be a result of post-transcriptional modification of hGSTP1-1 protein (62,63). Others have shown that hGSTP1 mRNA in MCF-7 cells is highly unstable relative to ER negative breast cancer cell lines (62). We have previously shown that hGSTT1-1 protein expression may be regulated in a similar manner in human colon cells (64). Alternatively, hypermethylation of GC rich regions within the hGSTP1 promotor also contributes significantly to the maintenance of hGSTP1-1 expression in MCF-7 (65). More recently, a proteasome-dependent regulatory mechanism of hGSTP1-1 protein turnover was reported (66). Thus, a higher proteasome activity in MCF-7 cells could possibly also account for the differences in basal expression of this GST in the two cell lines.

The lack of appreciable hGSTP1-1 protein and the weak expression of alpha and mu class GSTs in MCF-7 cells, all of which conjugate CDNB, is consistent with the low level of GST-CDNB activity in MCF-7 cells relative to the MCF-10A cells, although both cell lines had comparable levels of GSH. Our observations are supported by a study by Fields et al. (67) who also observed relatively low GST-CDNB activity in MCF-7 cells. As discussed, the presence of allelic and deletion variants as well as mRNA expression levels appear to be collectively crucial for the higher susceptibility of MCF-7 to cancer associated risk factors 4-HNE and BPDE (data not shown).

Our findings strongly support a general up-regulation of GSTs in non-neoplastic MCF-10A cells on exposure to genistein, with the possible exception that hGSTA4 mRNA expression was downregulated by genistein. Despite the negative effect of genistein on hGSTA4 mRNA expression, we found no modulation of hGSTA4-4 protein. An explanation for this discrepancy could be reduced mRNA stability, which we have previously reported for hGSTT1-1 in human colon cells exposed to butyrate and attributed to a shortened hGSTT1 mRNA half-life (64). The high correlation between hGSTP1-1 protein expression and GST-CDNB activity indicates that hGSTP1-1 induction is likely a major component responsible for the increase in GST-CDNB activity in MCF-10A cells. However, we could not exclude an additional induction of other GST isoenzymes which may have contributed to increased GST activity toward CDNB, or that have differing affinities toward CDNB.

The consequences of GST up-regulation in primary breast cells or cells in early stages of cell transformation could be a beneficial mechanism to mitigate the damaging impact toward carcinogenic compounds. Support for this hypothesis is provided from studies investigating nutrient related compounds (38,40,61,68). In this regard, nutritional status appears to be a key modulating factor in the generation and amelioration of toxic metabolites of relevance to breast cancer (69). Compounds of toxicological significance include the mutagenic 4-hydroxy-2-alkenal 4-HNE, which is formed during the physiological process of lipid peroxidation, leading to etheno- and propano-DNA adducts (70,71). 4-HNE is genotoxic in human colon cells (40,72) and may induce damage in the tumour suppressor gene TP53 via DNA adduct formation (42,73). Conversely, benzo(a)pyrene is a well-characterized PAH produced during food heating and a substrate for CYP 1A-enzymes which biotransform the parent compound to the mutagenic and carcinogenic BPDE (74). This compound induces DNA damage and mutations by forming DNA adducts (75). The genotoxic effects of 4-HNE and BPDE can be prevented by conjugation with GSH catalysed by GSTs (32). The GST isoenzyme hGSTA4-4 has an unusually high substrate specificity toward 4-HNE (51,76) whereas hGSTP1-1 conjugates both 4-HNE (77) and BPDE (78).

Our results of the chemoprotection experiments showed that 4-HNE genotoxicity was markedly reduced following genistein pretreatment in MCF-10A cells, whereas no modulation was observed in the tumour cell line MCF-7. 4-HNE is a high-affinity substrate for hGSTA4-4. Studies from one of our laboratories have demonstrated that hGSTA4-4 can be preferentially localized in the mitochondria relative to the

Fig. 3. Modulation of hGSTP1-1 protein expression after 48 h exposure to genistein in MCF-10A cells. Significant differences of genistein-induced effects and control values (0 μM) were calculated by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test (***P < 0.01). Data represents the means ± SD of four experiments. Shown below is a representative blot of one experiment.

only the highest concentration of genistein (30 μM) significantly reduced the genotoxic response of BPDE. In contrast, genistein was not chemoprotective against either 4-HNE or BPDE-induced genotoxicity in the MCF-7 tumour cell line.

Discussion

In the present study, we have demonstrated that genistein may act as a blocking agent in breast cancer via induction of GST. In this regard, an up-regulated detoxification system may facilitate the detoxification and/or elimination of ultimate carcinogens, thereby eliciting an inhibitory effect against the initiation of cancer and potentially reducing the risk of tumourigenesis (28). Support for this hypothesis on blocking agents in cancer chemoprevention is evidenced by several in vivo and in vitro studies (39,59–61). Our data also revealed different expression patterns of GST among the two cell lines, with higher overall GST levels in non-transformed MCF-10A cells. The extremely low expression of GST isoforms hGSTA4 and hGSTP1 in MCF-7 cancer cells is noteworthy. The lack of detectable hGSTP1-1 protein in the presence of low amounts of hGSTP1 mRNA may be a result of post-transcriptional modification of hGSTP1-1 protein (62,63). Others have shown that hGSTP1 mRNA in MCF-7 cells is highly unstable relative to ER negative breast cancer cell lines (62). We have previously shown that hGSTT1-1 protein expression may be regulated in a similar manner in human colon cells (64). Alternatively, hypermethylation of GC rich regions within the hGSTP1 promotor also contributes significantly to the maintenance of hGSTP1-1 expression in MCF-7 (65). More recently, a proteasome-dependent regulatory mechanism of hGSTP1-1 protein turnover was reported (66). Thus, a higher proteasome activity in MCF-7 cells could possibly also account for
cytosol (51), but can exist in phosphorylated forms in the cytoplasm of many cells and tissues (79,80). The reduction of 4-HNE genotoxicity in genistein treated MCF-10A cells, however, can not be explained by an enhanced GSTA4-4 activity, since its expression is relatively low in cells (32) and moreover, since it was not significantly modulated by genistein. In contrast, hGSTP1-1, which also may detoxify 4-HNE (77), was highly expressed in MCF-10A cells, and was induced by genistein. Collectively, these data support the hypothesis that hGSTP1-1 is a major enzyme involved in detoxification of 4-HNE in these non-neoplastic cells. This, despite the fact that we observed a discrepancy in the dose-response profiles, was associated with the effects of genistein on DNA damage and GST expression. Specifically, at relatively low and biologically relevant doses (1 μM) genistein inhibited DNA damage, whereas higher doses of 10 or 30 μM were needed to significantly modulate GST mRNA and protein levels, respectively. As discussed above, these observations could be due to the contribution of other HNE-detoxification pathways (e.g. aldehyde reductases, alcohol dehydrogenases) that were potentially modulated at lower doses of 1 μM genistein.

Increased GSTP1-1 activity may also be responsible for the reduction of BPDE-mediated DNA damage by genistein. The direct conjugation and detoxification of BPDE by hGSTP1-1, however, may only partially explain the protective role of genistein, since BPDE also has indirect modes of genotoxicity. Thus, in vitro studies have shown that comet DNA tailing effects induced by benzo(a)pyrene or other
bulky adduct-forming mutagens arise mainly from transient DNA single strand breaks formed during excision repair (81). This suggests that increased DNA excision repair by genistein could possibly contribute to the effect of GST-mediated metabolic deactivation. Previous cDNA macroarray experiments from our group confirmed a genistein-mediated up-regulation of XRCC repair genes (results not shown). Our conclusion is that the relatively high basal expression of hGSTP1-1 and associated high GST activity protected against 4-HNE and BPDE-induced DNA damage. This is consistent with a study by Fields et al. (82) where no protection against genotoxicity was seen at low GST expression and activity levels as shown in MCF-7 cells. Collectively, these studies indicate that a threshold level of GST activity may be required to achieve adequate protection against cellular damage by carcinogens.

Little is known about the exact underlying mechanisms of the modulation of hGSTP1 and hGSTA4 mRNA expression by phytoprotective compounds such as genistein. In fact, the regulation of GSTs is governed by a complex set of exogenous and endogenous parameters. In addition to dietary phytochemicals, other environmental factors such as exposure to carcinogens and endogenous metabolites such as ROS and lipid peroxidation products may modulate cellular detoxification capacities. The aforementioned compounds appear to confer resistance against carcinogenesis through their ability to generate a redox signal that stimulates increased expression of protective detoxification enzymes such as GSTs (32,83–86). However, it must be pointed out that the complex in vitro environment may not be reflected using the in vitro approaches with cell lines. Nevertheless, our studies do imply that the dietary phytoestrogens, especially genistein, can additionally improve the selected detoxification systems and thus may contribute to the prevention of human mammary carcinogenesis.

A potential mechanism to induce GST expression is thereby the activation of signalling pathways. Previous work in our laboratory indicated that the fermentation product butyrate activated MAP kinase cascade pathways by enhancing ERK1/2 phosphorylation in human colon cells (40). The activation of MAP kinase signalling pathways was also discussed for known phase II gene inducers such as phenolic antioxidants, isothiocyanates and phenolic flavonoids (87–89). Binding of AP-1 like transcription factors such as Jun, Fos and Nrf2 to the antioxidant responsive element-like sequences within the hGSTP1 promoter is a downstream consequence of ERK phosphorylation events (90–93).

In breast cells, there exist other ER-dependent and ER-independent signalling pathways for estrogens and phytoestrogens. In ER positive cells, the formed activated hormone receptor complex can bind directly to an ERE binding site within the promoter sequences of AP-1 forming transcriptions factors (94), or can bind directly to members of the AP-1 complex such as Jun, Fos, Nrf2 (37,89,95). ERα and ERβ exert differential actions on the AP-1 complex. If estrogens bind to ERβ the transcription via AP-1 will be inhibited, while the antiestrogen-ERβ complex activates the transcription via AP-1 (95). The high affinity of phytoestrogens for ERβ could be a possible activation pathway of GSTs by genistein. Another ER-mediated inductive mechanism is the indirect activation of MAP kinase cascades like ERK1/2 and JNK (96). Consequently, the resulting AP-1 complex activates hGSTP1 transcription. The ER-independent gene regulation is mediated by G protein-coupled receptors (GPR). Estradiol and genistein induce ERK1/2 phosphorylation via GPR30 (97) leading to activation of AP-1. Thus, hormones and phytohormones do not require ER expression and the MAPK pathway emerges as crucial.

It must be noted that the concentrations of genistein (1–30 μM) used in the present study were above isoflavone concentrations reported in plasma after dietary consumption of isoflavones (≤1 μM) (98). However, in the design of the present study, we took into account that short-term (24–72 h) exposure durations using the cell lines were of different duration than those in vivo situations in which more chronic dietary exposure of soy isoflavones results in low micromolar...
concentrations in serum. Moreover, continuous soy consumption throughout the life span may potentially result in accumulation of isoflavones in target tissues at levels exceeding those in serum. Several reports involving either single dose or short-term isoflavone treatments (including genistein) have shown increased plasma and tissue concentrations well above basal levels in respective control groups (99–104). Furthermore, infants exposed to dietary levels of 4.5–8.0 mg total isoflavones per kg body weight in soy-based formulas displayed plasma genistein and daidzein concentrations of 2.5 μM (105).

In conclusion, the results of the present study indicate that genistein mediates an enhancement of GST activity through induction of hGSTP1 mRNA and protein expression in human mammary non-neoplastic MCF-10A cells. Furthermore, modulation of GSTs on exposure to genistein leads to reduced genotoxicity on exposure to relevant proximate carcinogens, 4-HNE and BPDE. Our data are supportive in human mammary non-neoplastic MCF-10A cells. Further-

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References

Genistein modulates GSTs
