Differential induction of quinone reductase by phytoestrogens and protection against oestrogen-induced DNA damage

Nicole R. BIANCO, Laura J. CHAPLIN and Monica M. MONTANO¹

Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

Quinone reductase (QR) is a phase II detoxification enzyme that plays an important role in detoxifying quinones and may help maintain the antioxidant function of the cell. We have previously observed that QR is up-regulated by anti-oestrogens, but not oestrogen, in breast cancer cells via ER β (oestrogen receptor β) trans-activation. Such QR induction appears to protect breast cells against oestrogen-induced oxidative DNA damage, most likely by reducing reactive oestrogen metabolites termed catecholesterogen-quinones back to the hydroxy-catecholesterogens which may be conjugated. We now report that the phytoestrogens biochanin A, genistein and resveratrol also up-regulate QR expression in breast cancer cells. We observe that regulation can occur at the transcriptional level, preferentially through ER β transactivation at the electrophile response element of the QR gene promoter. By chromatin immunoprecipitation analysis, we show binding of ER α

and ER β to the QR promoter, with increased ER β binding in the presence of resveratrol. Functional studies show that biochanin A and resveratrol, but not genistein, can significantly protect against oestrogen-induced oxidative DNA damage in breast cancer cells. Antisense technology was used to determine whether such protection was dependent on ER β or QR. Our results with resveratrol are consistent with our hypothesis that the protective ability of resveratrol is partially dependent on the presence of ER β and QR. In conclusion, we postulate that phytoestrogen-mediated induction of QR may represent an additional mechanism for breast cancer protection, although the effects may be specific for a given phytoestrogen.

Key words: breast cancer, oestrogen, oestrogen receptor β , oxidative damage, phytoestrogens, quinone reductase.

INTRODUCTION

Quinone reductase (QR), or NAD(P)H:quinone oxidoreductase (EC 1.6.99.2), is a phase II detoxification enzyme that protects cells against reactive oxygen species generated by exogenous quinones and related compounds [1]. QR may also help to maintain the overall antioxidant functions of the cell by maintaining α -tocopherolquinone (vitamin E) and ubiquinones (coenzyme Q) in their reduced and active states [1]. For these reasons, potent inducers of QR are currently being studied in cancer chemopreventive studies [1,2].

Oestrogens, which are steroidal hormones (Figure 1), exert their effects on their target tissues by binding to their specific nuclear receptor, ER (oestrogen receptor) (reviewed in [3]). Once ER is bound to its ligand, it forms a homodimer and binds to DNA at specific sites called EREs (oestrogen response elements). The binding of the ER homodimer to DNA causes the recruitment of coactivators and the rest of the transcriptional machinery. This process results in the expression of genes that somehow bring about oestrogen's physiological actions, such as cell proliferation. Synthetic anti-oestrogens, which can be both steroidal and non-steroidal, can repress the transcriptional activity of ER. We have previously shown that QR may be transcriptionally activated by anti-oestrogen-bound ER, preferentially the ER β isoform [4]. Interestingly, there is no activation of QR by oestrogen-bound ER. Such regulation of QR may play an important role in breast cancer chemoprevention by anti-oestrogens such as tamoxifen [5–7].

Studies in our laboratory show that QR possesses an additional ability to protect against oestrogen-induced DNA damage in

breast cells [8]. In certain cell types, including breast, oestrogens may be hydroxylated by extrahepatic cytochrome P450 enzymes (mainly CYP1A1 and CYP1B1) to hydroxy-catecholesterogens, and further oxidized to the semiquinone and quinone form [9,10]. This metabolism is potentially harmful, given that the quinone-catecholesterogen can bind to DNA and form DNA adducts; and redox cycling between the quinone and semiquinone form causes superoxide formation that could lead to hydroxylated nucleotide bases. It has been postulated that oestrogen-induced DNA damage and oxidative stress are involved in the initiation of breast cancer [10–12]. The ER may then provide a selective proliferative advantage to these damaged cells, possibly leading to tumour formation. We have recently shown that up-regulation of QR, by anti-oestrogens or over-expression, prevents E₂ (oestradiol)-induced oxidative DNA damage in breast cells [8]. The mechanism for QR protection most likely revolves around its ability to reduce reactive quinone-catecholesterogens back to the hydroxy-catecholesterogens, which are then detoxified through conjugation and excretion [13,14]. This would prevent oxygen free radical formation from redox cycling of the quinone and semiquinone catecholesterogens, as well as direct reaction of the quinone-catecholesterogens with DNA.

We sought to determine whether additional, more 'natural' ligands would also regulate QR expression and display protective effects against E₂-induced DNA damage. This led us to examine regulation by phytoestrogens. Phytoestrogens are naturally occurring weak oestrogens found in a wide variety of plant products, such as fruits, vegetables, grains and soya beans [15,16]. The most studied classes include the isoflavones and lignans, and they all share a similar chemical structure to oestrogen (Figure 1).

Abbreviations used: Bio A, biochanin A; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; E₂, oestradiol; EpRE, electrophile response element; ER, oestrogen receptor; ER β AS, ER β antisense; Gen, genistein; NAC, N-acetylcysteine; 8-OHdG, 8-hydroxydeoxyguanosine; QR, quinone reductase; QRAS, QR antisense; Res, resveratrol; SERM, selective oestrogen receptor modulator; TBHQ, t-butylhydroquinone; TK, thymidine kinase.

¹ To whom correspondence should be addressed (email mxm126@po.cwru.edu).

Figure 1 Chemical structures of E₂ and the phytoestrogens: Res, Bio A and Gen

Genistein (Gen) and biochanin A (Bio A) are isoflavones found mainly in soya beans and red clover respectively, whereas resveratrol (Res) is a stilbene found in grape skins. Phytoestrogens have been shown to generally protect against chemically and virally induced breast cancer in mouse and rat models [17,18]. Phytoestrogen chemoprevention in humans has yet to be confirmed [18,19]. Like oestrogens, phytoestrogens exert their effects through the ER. Many phytoestrogens show preferential binding and transcriptional activity through ER β [20–23]. While the biological effects of phytoestrogens are generally 10 000-fold weaker than oestrogen, their levels in the body can exceed oestrogen levels by 10 000-fold, making their presence physiologically relevant [18].

Several phytoestrogens induce QR activity in human cancer cells, although until now the mechanism had not been determined [24–26]. On the basis of the above-mentioned similarities to our model for anti-oestrogen-ER β regulation of the QR promoter and the apparent cancer-protective effects of phytoestrogens, we examined the phytoestrogens Bio A, Gen, and Res for mechanistically similar activation of QR and effects on oestrogen-induced DNA damage.

Gen, Bio A and Res all induce increases in QR at the protein level in breast cancer cells. Transcriptional studies show such regulation can occur at the EpRE (electrophile response element) through ER β transactivation. We show that ER α and ER β bind to the QR promoter *in vivo*, with increased ER β binding in the presence of Res. Moreover, two of the phytoestrogens, Bio A and Res, significantly protect against oestrogen-induced oxidative DNA damage, as measured by 8-OHdG (8-hydroxydeoxyguanosine) levels. Also, at least for Res, this protection appears to be related to up-regulation of QR through ER β , as knock-down of QR and ER β diminishes the protective effect. Regulation of detoxification genes, such as those expressing QR, may partially explain the protective effects that certain phytoestrogens have in breast and other cancers.

EXPERIMENTAL

Chemicals and materials

Cell culture media was purchased from Gibco (Grand Island, NY, U.S.A.). Calf serum was from Hyclone Laboratories (Logan, UT, U.S.A.) and fetal calf serum from Atlanta Biologicals (Norcross, GA, U.S.A.). E₂, Gen, Bio A, Res, TBHQ (t-butylhydroquinone) and NAC (*N*-acetylcysteine) were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell culture and transfections

MCF7 and MDA-MB-231 cells were maintained and transfected as described previously [27]. MDA-MB-231 cells were depleted of oestrogen by growth in improved minimal essential media without phenol red containing 5% charcoal dextran-treated calf serum for 5 days before experiments. Cells seeded in 60-mm dishes were transfected as previously described [28] using 4 μ g of the EpRE-tk-luciferase reporter constructs, 100 ng of pCMV5-ER expression vector and 0.15 μ g of pRL-CMV internal control plasmid (Promega, Madison, WI, U.S.A.). Luciferase activity was measured using the Dual-Luciferase[®] Reporter Assay System kit (Promega) on an Lmax[™] microplate luminometer (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.).

Northern blot analyses

Total RNA was isolated using Trizol (Gibco). Gel-purified re-amplified QR cDNA was random-primer labelled using the Ready-to-Go DNA labelling kit from Pharmacia (Piscataway, NJ, U.S.A.) for Northern analysis. Total RNA (20 μ g) was separated by electrophoresis, transferred on to nitrocellulose support, and hybridized with random-primer-labelled cDNA. Quantitative analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

Western blot analyses

Whole cell extracts were prepared from breast epithelial cells using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, U.S.A.). Protein extract (25 μ g) was separated by electrophoresis on SDS/12% polyacrylamide gels and transferred by electrophoresis on to nitrocellulose membranes. Blots were incubated overnight at 4°C with QR polyclonal antibody (1:3000) and horseradish-peroxidase-conjugated anti-rabbit secondary antibody (1:3000) for detection by chemiluminescence (ECL[®], Amersham Pharmacia). The QR antibody was produced for our laboratory by Biosynthesis (Lewisville, TX, U.S.A.) using a peptide representing amino acids 83–98 of human QR. To control for unequal protein loading, blots were then stripped of antibody using Re-Blot Plus Mild Antibody Stripping Solution (Chemicon International, Temecula, CA, U.S.A.) according to the manufacturer's protocol, and re-blotted with cytokeratin 18 (DC-10) monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (1:2000; Chemicon International). ER α was detected using the ID5 monoclonal antibody (Lab Vision, Fremont, CA) and a horseradish-peroxidase-conjugated goat anti-mouse IgG secondary antibody (Pierce). Western blot analyses for ER β have been described previously [9]. Quantitative analysis was performed using the NIH Image program.

ChIP (chromatin immunoprecipitation) assay

MCF7 cells which had been depleted of oestrogen were treated for 45 min with phytoestrogens (1 μ M) or vehicle (ethanol). The cells were then cross-linked by the addition of 1% formaldehyde to the plates and rotated for 10 min at 37°C. The cross-linking reaction was quenched with 0.125 M glycine and rotated for 5 min at 37°C. Cells were pelleted, rinsed with PBS and resuspended twice in 1 ml of cell lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl and 0.5% Nonidet P40) containing protease inhibitors. Cells were pelleted and then resuspended in nuclei lysis buffer (50 mM Tris/HCl, pH 8.0, 10 mM EDTA and 1% SDS) containing protease inhibitors. Chromatin was sonicated to an average size of 300–1000 bp using a tapered microtip on a Sonifier 450 using 7 $\times$ 10 s bursts with a rest period of 1 min in between. Sheared chromatin (50 μ l) was then diluted in 950 μ l of IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.0, 167 mM NaCl) and precleared for 1 h at 4°C with 80 μ l of a Protein A/salmon sperm DNA/tRNA mixture (1 ml of Protein A slurry, 24 μ l of 10 mg/ml salmon sperm DNA, 24 μ l of 10 mg/ml tRNA). The supernatant was incubated overnight at 4°C with 5 μ g of antibody. Antibodies for ER α , ER β , Nrf2 and MafK were obtained from Santa Cruz Biotechnologies. For negative controls, either non-specific or no primary antibody was used. To pull-down the antibody–protein–DNA complexes, samples were incubated at 4°C for 1 h with 60 μ l of the Protein A/salmon sperm DNA/tRNA mixture. Protein A beads were centrifuged and washed twice for 10 min each with the following four buffers at 4°C: IP dilution buffer, TSE-500 wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.0, 500 mM NaCl), LiCl/detergent wash (100 mM Tris/HCl, pH 8.0, 500 mM LiCl, 1% Nonidet P40, 1% deoxycholic acid), and finally TE. The complex was eluted from the Protein A beads with 2 $\times$ 250 μ l of elution buffer (50 mM NaHCO₃, 1% SDS) at room temperature (25°C) for 15 min, and reverse cross-linked by the addition of 20 μ l of 5 M NaCl and incubation overnight at 65°C. Samples were treated with proteinase K and RNase at 45°C for 1 h, phenol extracted, ethanol precipitated and resuspended in 20 μ l of water. PCR conditions were optimized using the PCR Optimization kit from Roche (Mannheim, Germany). The PCR primers used are as follows: for the EpRE, QR(–591/–567), 5'-CATGCACCCAGGGAAGTGTGTGT-3', QR(–393/–417) 5'-GCACGAAATGGAGCAGAAAAAGAG-3'; for the control, QR(–1631/–1611), 5'-TTTGCCTCAGATCCTGAGT-A-3', QR(–1315/–1334), 5'-TGATGCAACAAGAGAGGAC-3'.

Immunocytochemistry for 8-OHdG in breast cells

This procedure has been described in detail previously [8]. Cells grown on coverslips were fixed in methacarn (methanol/chloroform/acetic acid, 6:3:1) for 1 h at room temperature. Endogenous peroxidase activity in the cells was eliminated by a 30 min incubation with 3% H₂O₂ in methanol, and non-specific binding sites were blocked in a 15 min incubation with 10% normal goat serum in Tris-buffered saline (150 mM Tris/HCl and 150 mM NaCl, pH 7.6). The cells were then pretreated with proteinase K (20 μ g/ml in PBS, pH 7.4, for 15 min at room temperature). To detect oxidized nucleosides, we used the anti-8-oxo-dG monoclonal antibody 1F7 (1:100; Trevigen, Gaithersburg, MD). As a negative control, cells were incubated without the primary antibody. Immunostaining was developed by the peroxidase–anti-peroxidase procedure.

Relative quantification of 8-OHdG

Immunoreactivity was evaluated by measuring absorbance (A), as described previously [8]. The absorbance was assessed using a

Carl Zeiss Axiocam digital camera with a KS300 Imaging System quantification program. The absorbance of manually outlined cells was measured. Five cells in three adjacent fields were measured and the background absorbance was subtracted from each. Each experiment was performed three or more times and results were measured under the same optical and light conditions. Also, an electronic shading correction was used to compensate for any unevenness that might be present in the illumination. Statistical analysis was performed using the Student's *t* test or ANOVA.

Retroviral-mediated transfection

Retroviruses were made by transfecting PA317 cells with the pBPSTR1 plasmid alone, pBPSTR1 containing antisense QR or ER β , or pBPSTR1 containing sense ER α or ER β . Construction of pBPSTR1 sense and antisense plasmids and retroviruses has been described previously [9]. Breast epithelial cell lines were infected with retrovirus-containing supernatants in the absence or presence of 3 μ g/ml tetracycline. The self-contained, tetracycline-regulated retroviral vector pBPSTR1 contains both the response unit, comprising tetracycline resistance operon regulatory elements (*tetO*) within a minimal CMV (cytomegalovirus) promoter, and the regulator unit, encoding the tTA protein (the tetracycline repressor fused to the transactivator protein VP16) [29]. Gene expression is inhibited by tetracycline, which binds the transactivator protein tTA, causing it to dissociate from the *tetO* minimal CMV promoter. Changes in QR, ER α or ER β protein expression were verified by immunofluorescence staining [8]. Changes in protein levels were also verified in MCF7 cells by Western blot analysis (results not shown).

RESULTS

Phytoestrogens induce QR transcription and expression in breast cancer cells

Although previous studies have reported phytoestrogen induction of QR, both at the mRNA and protein levels, such studies have not been performed in breast cancer cells, nor has a mechanism for regulation been proposed [24,25]. Thus we have examined three phytoestrogens, Bio A, Gen, and Res, for possible regulation of QR in cultured breast cancer cells. Figure 1 shows the chemical structures of these compounds and their similarity to E₂.

MDA-MB-231 (no ER α , low ER β) and MCF7 (ER α / β positive) breast cancer cells [30] were treated with Bio A, Gen, and Res to measure changes in QR mRNA and protein levels. The cells were chosen on the basis of their low levels of endogenous QR expression. We have previously reported similar expression of ER β in MDA-MB-231 and MCF7 cells using Western blot analyses [8]. As expected, ER α was detected in MCF7, but not MDA-MB-231 cells. In MDA-MB-231 breast cancer cells over-expressing ER β , we observed increases in QR mRNA levels by Northern blot analyses after 24 h of Gen and Res treatment (*P* < 0.05, Figure 2A). Induction by Bio A was not evident after normalization to the rRNA band. Protein levels of QR were increased approx. 2–5-fold after 48 h of treatment with all three phytoestrogens in MDA-MB-231 cells over-expressing ER β and MCF-7 cells, as measured by Western blot analysis (Figures 2B and 2C). The values below each blot represent relative protein levels after normalization to cytokeratin 18 or glyceraldehyde-3-phosphate dehydrogenase levels. Also, induction is comparable with or higher than the positive control TBHQ (10 μ M), a known transcriptional activator of QR. These levels correlate similarly with induction of QR activity by Gen (7-fold) and Bio A (3-fold)

Figure 2 Differential phytoestrogen induction of QR mRNA and protein levels

(A) Northern blot analysis. MDA-MB-231 breast cancer cells over-expressing ER β were treated for 24 h with vehicle control (C) or 10 μ M Bio A, Gen or Res. Total RNA was collected and equal amounts (20 μ g) of total RNA were separated by electrophoresis and transferred on to a nylon membrane. The blot was then probed with random-primer-labelled QR cDNA. The 18 S ribosomal band was used to normalize for unequal loading. The values below the blot represent the average fold induction $\pm$ S.E.M. from three experiments. (B) and (C) Western blot analysis. Whole cell extracts were prepared from MCF7 (B) or MDA-MB-231 (C) breast cancer cells treated for 48 h with the indicated concentrations of Bio A, Gen, Res or TBHQ. Protein extracts were separated by electrophoresis on SDS/12% polyacrylamide gels and transferred on to nitrocellulose filters. The blot was then incubated with QR polyclonal antibody and horseradish-peroxidase-conjugated secondary antibody and revealed by chemiluminescence. The membrane was re-blotted with cytokeratin 18 (Cyto 18) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody to normalize for unequal protein loading. The values below the blot represent the average fold induction $\pm$ S.E.M. from 3–5 experiments. For (A–C), * P < 0.05 as determined by the Student's t test.

in colon cancer cells [24], and a 1.9-fold induction by Res in mouse hepatoma cells [26]. Measurable increases in QR protein, but not mRNA, by Bio A suggests an additional level of control at the translational level by this compound.

Anti-oestrogen regulation of the QR gene requires the EpRE of the QR promoter [27]. Transfection assays using the EpRE-luciferase reporter gene (EpRE-tk-pGL3) in MDA-MB-231 breast cancer cells over-expressing ER β revealed a dose-dependent induction of EpRE activity by all phytoestrogens. The induction was significantly greater than when ER β was over-expressed in the absence of ligand (Figure 3).

Figure 3 EpRE-mediated transcriptional activation by phytoestrogens

MDA-MB-231 breast cancer cells were co-transfected with an expression construct for ER β along with the reporter gene EpRE-tk-pGL3 and treated for 48 h with the indicated doses of Bio A, Gen, Res or the positive control TBHQ (10 μ M). The cells were also transfected with a *Renilla* luciferase internal control plasmid to normalize for transfection efficiency. Luciferase activity was measured, and fold activation over vehicle treatment of cells not transfected with ER β is displayed as the means $\pm$ S.E.M. for three independent experiments. * P < 0.05 and ** P < 0.001 when compared with ER β over-expressing cells treated with vehicle alone as determined by the Student's t test.

Figure 4 ER β is the isoform critical for EpRE activation

MDA-MB-231 breast cancer cells were co-transfected with control expression vector (no ER α or ER β cDNA) or an expression vector for ER α or ER β along with the reporter gene EpRE-tk-pGL3 and treated for 48 h with the indicated concentrations of Bio A, Gen or Res. The cells were also transfected with a *Renilla* luciferase internal control plasmid to normalize for transfection efficiency. Luciferase activity was measured, and fold activation over cells not transfected with ER α or ER β is displayed as an average $\pm$ S.E.M. for three independent experiments. * P < 0.05 when compared with control cells or cells expressing ER α with the same treatment as determined by ANOVA.

Phytoestrogen regulation of QR gene transcriptional activity by ER β

Phytoestrogens, including Bio A and Gen used in the present study, bind preferentially to the ER β isoform [22]. Res, however, binds to ER α and ER β with similar affinity [31]. To determine which isoform is mediating the phytoestrogen induction of QR, we co-transfected an expression plasmid for ER α or ER β , together with the EpRE-tk-pGL3 reporter plasmid, into MDA-MB-231 cells. All three phytoestrogens induced the EpRE to the highest levels when ER β was over-expressed. However, only Res activation of the reporter gene when ER β is over-expressed was considered statistically greater than when control vector is transfected or when ER α is over-expressed (P < 0.05, Figure 4). Activation by Bio A (10 μ M) of ER β was higher than ER α , but the difference was not statistically significant (P = 0.056). Activation in the absence of ER may be due to low levels of endogenous ER β in our MDA-MB-231 cells [8]. These results suggest that

Figure 5 Ligand-dependent binding of ER to the QR promoter

(A) Sonicated chromatin extracts from MCF7 cells treated for 45 min with vehicle (Φ , lane 1), 1 μ M Bio A (B, lane 2), Gen (G, lane 3), Res (R, lanes 4–6 and 8) were incubated with antibody to ER α or ER β . Negative controls were incubated with either non-specific rabbit IgG (lane 5) or no primary antibody (lane 6). As a positive control, chromatin extracts were incubated with Nrf2 antibody (row 3). Bound QR promoter-containing chromatin was detected by PCR analysis using a set of primers surrounding the EpRE (lanes 1–6) or control primers to a region upstream of the EpRE (lanes 7–8). Input chromatin (2%) was also analysed by PCR for a comparison control (row 4). (B) ChIP assays were performed as in (A) using extracts from cells treated for 45 min with vehicle (Φ , lane 1), 1 μ M Res (R, lane 2) or 10 μ M TBHQ (TQ, lane 4) and incubated with antibody against MafK or ER β . (C) ChIP assays were performed as in (A) using extracts from cells treated for 45 min with vehicle (Φ , lane 1), 1 μ M Gen (G, lane 2), 15 mM NAC (lane 3) or Gen and NAC (G + NAC, lane 4), and incubated with antibody against ER β or NRF2. The results shown in (A–C) are representative of three or more separate experiments. (D) MDA-MB-231 breast cancer cells were transfected with an expression vector for ER β along with the reporter

phytoestrogen activation of the EpRE occurs primarily through ER β , which is also seen with anti-oestrogen regulation [4].

ER binding to the EpRE region of the QR promoter

ER α and ER β are capable of interaction with the EpRE based on results from *in vitro* gel mobility-shift assays, although other proteins are most likely involved [4,32]. In the present study, we wanted to verify this interaction using a more *in vivo* approach and to determine ligand dependence of binding. The ChIP assay allows for cellular detection of transcription factor binding to any DNA regulatory region of interest. Using polyclonal antibodies for ER α or ER β , we are able to precipitate sonicated chromatin DNA from the EpRE-containing region of the QR promoter, as detected by PCR analysis. The PCR primers are specific for the QR promoter and yield a 200-bp product flanking the EpRE. MCF7 cells were chosen because they contain both ER isoforms [33]. MCF7 breast cancer cells were treated with vehicle or phytoestrogens (1 μ M) for 45 min, because this is the optimal timing for ER binding to an oestrogen response element in MCF7 cells [34]. In addition, preliminary studies showed this to be the optimal timing for ER binding to the EpRE, as there was no binding at 15 or 90 min of treatment (results not shown).

With vehicle alone, there is a similar basal level binding of both ER α and ER β (Figure 5A). However, in 3 out of 4 independent experiments, there was a consistent increase in ER β binding in the presence of Res when compared with vehicle alone. ER α showed no consistent differences in binding with the various ligands. Thus ER ligand-specific regulation of the EpRE may be partially regulated by ER binding, at least when Res is the ligand. Nrf2 is a b-zip (leucine zipper) transcription factor that positively regulates EpRE-mediated transactivation of the QR gene [35]. We used Nrf2 in the present study as a positive control to show the assay was working properly. In addition ER may regulate EpRE enhancer activity by modulating Nrf2 recruitment to the EpRE. In repeated experiments, there was no discernible difference in Nrf2 binding in the presence of the various ligands. Alternatively, ER may regulate QR gene transcription by modulating binding of small Maf (musculoaponeurotic fibrosarcoma virus) protein, such as MafK, to the EpRE. MafK has been shown to interact with the EpRE and repress its activity [36]. However, we observed no change in MafK binding, relative to control, in the presence of resveratrol, which we have shown to induce increased ER β binding (Figure 5B, lanes 1 and 2). In the presence of TBHQ, a well-known inducer of EpRE enhancer activity, we did not see a change in ER β binding when compared with vehicle alone (Figure 5B, lanes 3 and 4). This is consistent with our previous finding that ER is not necessary for TBHQ-mediated induction of QR transcriptional activity [4].

As a negative control we show that there were no PCR bands observed with normal rabbit IgG or in the absence of antibody (Figure 5A, lanes 5 and 6). To verify the selectivity of ER for the EpRE-containing region of the QR promoter, we also performed the PCR using a different set of primers that are approx. 1 kb from the EpRE primers. ER β , in the presence of Res, did not bind to this region of the QR promoter (Figure 5A, lanes 7 and 8).

Gen, or its metabolites, have been reported to have genotoxic effects in breast cells [37]. We therefore determined if Gen-induced DNA damage is involved in redox regulation of Nrf2 [38],

gene EpRE-tk-pGL3 and treated for 48 h with the vehicle, 10 μ M Gen and/or 10 mM NAC. The cells were also transfected with a *Renilla* luciferase internal control plasmid to normalize for transfection efficiency. Luciferase activity was measured, and fold activation over cells treated with vehicle is expressed as the average $\pm$ S.E.M. for three independent experiments.

Figure 6 Phytoestrogens protect against E₂-induced oxidative DNA damage

MCF7 cells grown on coverslips were treated with vehicle (control) or E₂ (10 nM) with or without the phytoestrogens Bio A, Gen or Res at 10 μ M for 24 h, and immunostained for 8-OHdG. The relative levels of 8-OHdG after quantification are shown. The results are expressed as the means $\pm$ S.E.M. for three adjacent fields from three or more separate experiments. a, $P < 0.05$ versus control; b, $P < 0.05$ versus E₂ alone; as determined by the Student's *t* test.

inducing Nrf2 nuclear translocation, EpRE binding and perhaps recruitment of ER β . Alternatively, the ER appears to be subject to redox regulation, and the oxidized ERs show decreased DNA binding [39]. It is thus possible that Gen-induced oxidation of ER will inhibit ER binding to the EpRE. We used an antioxidant, NAC, that we have previously shown to inhibit oestrogen-induced DNA damage in MCF7 cells [8]. The 45 min time-point selected in ChIP assays has been previously shown to be sufficient for Gen metabolism and induction of DNA damage [37]. We did not see a change in Nrf2 or ER β binding when NAC was co-administered with Gen compared with Gen treatment alone (Figure 5C). Similarly NAC did not affect Gen induction of EpRE reporter gene activity (Figure 5D).

Phytoestrogen protection of oestrogen-induced DNA damage

We have previously demonstrated that anti-oestrogens can protect breast cells from oestrogen-induced oxidative DNA damage [8]. This is potentially important for the cancer-protective mechanism of anti-oestrogens, as DNA damage induced by oestrogens could potentially lead to tumour formation in susceptible tissues. Phytoestrogens are generally considered to be cancer protective as well, although the mechanism is still under study. If anti-oestrogens protect against oestrogen-induced DNA damage by up-regulation of detoxification genes such as QR, as we hypothesize, then phytoestrogens may possess this same ability. To test this, MCF7 cells were treated with oestrogen (10 nM) with or without the phytoestrogens (10 μ M) for 24 h. Oxidative DNA damage was assessed by quantitative immunocytochemistry of 8-OHdG, a commonly used marker. Interestingly, all three phytoestrogens at this dose are able to protect against oestrogen-induced 8-OHdG, although only Bio A and Res showed statistically significant protection ($p < 0.05$; Figure 6). Gen treatment alone induced a slight increase in 8-OHdG levels.

According to our hypothesis, phytoestrogens may partially exert their protective effects against oxidative DNA damage by binding to the ER and regulating the expression of detoxification

enzymes such as QR. Thus ER β and QR should be key players in this process. To determine the necessity of these two factors, we transiently reduced their expression in MCF7 cells using the self-contained tetracycline-regulated pBPSTR1 vector [29] containing antisense ER β and QR cDNA. Using the same batch of retroviruses, we observe an approx. 40–50 % reduction in protein level when measured by Western blotting (results not shown) or immunofluorescence [8].

When ER β protein levels were reduced, there was a general increase in 8-OHdG levels, even with vehicle-treated cells (Figure 7A). Also, Res was significantly less protective against E₂-induced 8-OHdG, when compared with mock-infected cells, suggesting that Res requires ER β for this effect. As for Bio A, it has only slightly impaired protective ability in the absence of ER β . Finally, Gen shows the opposite effect, being more protective in the absence of ER β . Thus the role of ER β in mediating the protective effects against oxidative DNA damage may depend upon the particular phytoestrogen.

As seen before, reduction of QR in breast cancer cells also causes an increase in 8-OHdG (Figure 7B) [8]. Also, when compared with the mock-infected cells (pBPSTR1 vector alone), Bio A and Res are significantly less protective ($P < 0.05$). Again, Gen shows the opposite effect, being more protective with reduced QR levels. At least for Bio A and Res, the protective effects are related to QR levels in the cell, suggesting QR to be an important part of the pathway. However, there may be additional detoxification enzymes regulated by Bio A and Res that may be able to compensate for the reduction of QR.

The differential effects of ER β _{AS} (ER β antisense) or QR_{AS} (QR antisense) on the protective effects of the different phytoestrogens may be attributed to less than optimal inhibition of ER β or QR. One argument against this is the increase in 8-OHdG levels in the E₂ plus Res group after ER β _{AS} retroviral infection, which is comparable with the increase in 8-OHdG levels observed after E₂ treatment of control cells. The same comparable increase is observed in the QR_{AS}-infected cells after E₂ plus Bio A treatment. In other words, the partial inhibition of QR or ER β is enough to produce complete abrogation of Bio A or Res protective effects respectively.

To further verify the role of ER β in phytoestrogen-mediated protection against E₂-induced DNA damage, we expressed ER α or ER β in MCF10A cells. We have previously reported that MCF10 cells do not express ER α or the full-length form of ER β , and *trans*-hydroxytamoxifen did not protect against E₂-induced DNA damage [8]. We now observe that MCF10A cells infected with control retroviruses or ER α retroviruses do not show inhibition of E₂-induced DNA damage by Res (Figure 7C). However, after infection with ER β retroviruses, Res is able to protect against E₂-induced DNA damage.

DISCUSSION

The results of the present study support the hypothesis that certain phytoestrogens may protect against potential cancer-causing 'hits' or mutations by inducing the expression of important detoxification genes, such as QR. Specifically, binding of phytoestrogens to ER β induces transcription of the QR gene. We have shown for the first time that this regulation is mediated by the EpRE of the QR promoter, as determined by reporter gene assays and ChIP. To our knowledge, this is also the first *in vivo* detection of ER binding to a non-classical oestrogen response element. Most importantly, concentrations of Bio A and Res at 1 μ M, similar to peak serum and tissue levels of other phytoestrogens [18,40], are able to protect against E₂-induced oxidative DNA damage (Figure 6).

Figure 7 Phytoestrogen protection in relation to ER β and QR levels

(A) MCF7 cells were transiently infected with ER β_{AS} retroviruses or control retroviruses. The cells were then treated with vehicle (control) or E₂ (10 nM) with or without the phytoestrogens Bio A, Gen or Res (1 μ M) for 24 h, and immunostained for 8-OHdG. The relative levels of 8-OHdG after quantification are shown. * P < 0.05 versus similarly treated control cells as determined by ANOVA. (B) MCF7 cells were transiently infected with QR_{AS} retroviruses or control retroviruses. The cells were then treated with vehicle (control) or E₂ (10 nM) with or without the phytoestrogens Bio A, Gen or Res (1 μ M) for 24 h, and immunostained for 8-OHdG. The relative levels of 8-OHdG after quantification are shown. * P < 0.05 versus similarly treated control cells as determined by ANOVA. (C) Upper panel: MCF10A cells were transiently infected with control retroviruses, ER α retroviruses or ER β retroviruses. The cells were then treated with vehicle (control) or E₂ (10 nM) with or without Res (1 μ M) for 24 h and immunostained for 8-OHdG. Shown are the relative levels of 8-OHdG after quantification. * P < 0.05 versus E₂ alone as determined by ANOVA. The results are expressed as the means $\pm$ S.E.M. of three adjacent fields from three or more separate experiments. Lower panels: Western blot analyses for ER α or ER β in MCF10A cells infected with control, ER α or ER β retroviruses. NSB is a non-specific band observed in the Western blot.

At least in the case of Res, this protection relies on ER β and QR, as reduction in the levels of these two proteins diminishes the protective effect. These results mimic those seen with the anti-oestrogen *trans*-hydroxytamoxifen [8]. Therefore, on the basis of our studies, we hypothesize that Res may be a suitable candidate for future studies regarding breast cancer chemoprevention.

While we have observed ER binding to the EpRE, it should be noted that the ChIP assay does not determine direct or indirect binding to the EpRE through protein–protein interactions. Although we did find differential binding of ER β to the EpRE in the presence of Res, there may also be ligand-specific binding of additional downstream ER co-activators, such as SRC-1 (steroid receptor coactivator-1) or GRIP1 (glucocorticoid-interacting receptor protein 1) that regulate transcription. It is also possible that the regulation may be occurring by another unknown factor that is not part of the ER complex. The similarities to anti-oestrogen up-regulation of QR are reinforced by crystallographic studies showing that the ER β ligand-binding domain in the presence of Gen adopts a conformation very similar to that of anti-oestrogen-bound ER β [41].

Interestingly, Gen induced QR expression, but gave no significant protection against E₂-induced oxidative DNA damage. Also, Gen by itself produces a small amount of 8-OHdG in the cells in our study (Figure 6). The existing literature on Gen genotoxicity is fairly controversial, as well as with the other phytoestrogens. Some reports favour the antioxidant ability of Gen both *in vitro* and *in vivo*. For example, Gen potently scavenges reactive oxygen species *in vitro*, preventing 8-OHdG formation [42]. Pretreatment of hairless mice with Gen also protects against UV B-induced 8-OHdG and skin tumorigenesis [43]. However, in a rat carcinogenesis model, Gen increased levels of 8-OHdG in the rat lung tissues [44]. This suggests, perhaps, a cell-type specific effect. One possibility is that Gen, similar to E₂, is metabolized to an unstable compound in certain cells that may generate reactive oxygen species. However, metabolic studies of Gen in MCF7 cells revealed the conjugated Gen 7-sulphate to be the major metabolite, although a hydroxylated and methylated metabolite was also present in small amounts [45]. If this metabolite is hydroxylated at the 3' position of the B ring (Figure 1), which occurs in rat liver microsomes [46], this would form a catechol that could generate oxygen radicals through formation of the semi-quinone and quinone catechols. In this case, the presence of QR may actually perpetuate the redox cycling and generation of oxygen radicals, as Gen catechol metabolites appear to be poor substrates for catechol-*O*-methyltransferase [47]. More recently, Murata et al. [37] reported that Gen metabolites induced DNA damage in breast epithelial cells. This may partially explain the result that Gen seems to appear more protective in QR_{AS}-containing cells. Interestingly, significant Gen metabolism is not observed in cultured normal mammary epithelial cells [45]. The observation that Gen becomes more protective in the absence of ER β suggests that ER β bound to Gen regulates an additional set of genes or proteins that leads to an increase in the oxidative stress of the cell.

Gen genotoxicity may lead to redox regulation of either ER β or Nrf2. It is possible that Gen can induce oxidation of the highly conserved cysteines in ER, which has been shown by others to inhibit ER–DNA interaction [38]. Several studies have shown that Nrf2 activity is up-regulated in response to oxidative stress (reviewed in [37]). However, we did not see a change in Nrf2 or ER β binding in the presence of Gen and the antioxidant NAC when compared with Gen alone.

Because Bio A contains a methoxy group at the 4' position, such a catechol would not form, unless demethylated to Gen first,

which does occur in MCF7 cells [45]. Thus determining the exact metabolism and reactivity of phytoestrogens in various tissues is necessary for predicting their cancer protective effects.

The possible significance of phytoestrogen regulation of QR is enhanced when one considers additional phase II detoxification genes that also contain EpREs in their regulatory regions. These other genes include γ -glutamylcysteine synthetase, glutathione S-transferase and UDP-glucuronosyl transferase [35]. The potential regulation of other detoxification genes is consistent with our observation that Bio A protects against E₂-induced 8-OHdG at 1 μ M, even though there is very little induction of QR protein at this concentration in MCF7 cells (results not shown). Future studies in our laboratory will address these possibilities.

There are also many additional protective mechanisms for phytoestrogens that have been proposed in the literature (reviewed in [48]). These include: (1) antioxidant scavenging of free radicals, (2) inhibition of ER activity by acting as a weak antagonist, (3) inhibition of angiogenesis and metastasis, (4) inhibition of protein tyrosine kinases important for growth signalling, (5) inhibition of topoisomerase I and II, (6) inhibition of NF- κ B (nuclear factor κ B) and Akt stress signaling pathways, (7) induction of differentiation of breast cells, and (8) the reduction of available E₂ through various proposed mechanisms. It is also possible that each phytoestrogen may have various combinations of mechanisms. The varied results shown here illustrate this possibility. A better understanding of the mechanism of phytoestrogen protection should allow for tailored dietary supplementation of phytoestrogens to help protect against malignancies such as breast and prostate cancer.

We would like to extend our appreciation to the members of the George Perry and Mark A. Smith laboratories [Institute of Pathology, CWRU (Case Western Reserve University)] for their technical assistance, advice and the use of their microscope for the 8-OHdG studies. We thank Dr Mireya Diaz (Department of Epidemiology and Biostatistics, CWRU) and Jonathan Mosely (Department of Pharmacology, CWRU) for help with statistical analyses. We also thank Dr Steven A. Reeves (Massachusetts General Hospital) for the pBPSTR1 retroviral vector and Dr Anil Jaiswal (Baylor College of Medicine) for the pMT2-QR vector. This work was supported by National Institutes of Health grants (CA80959 and CA92440) to M.M.M. and Institutional NRSA Predoctoral Fellowships (T32 GM08056 and 5T32 CA59366-CA59310) to N.R.B.

REFERENCES

- Talalay, P. and Dinkova-Kostova, A. T. (2004) Role of nicotinamide quinone oxidoreductase 1 (NQO1) in protection against toxicity of electrophiles and reactive oxygen intermediates. *Methods Enzymol.* **382**, 355–364
- Begleiter, A. and Fourie, J. (2004) Induction of NQO1 in cancer cells. *Methods Enzymol.* **382**, 320–351
- Barkhem, T., Nilsson, S. and Gustafsson, J. A. (2004) Molecular mechanisms, physiological consequences and pharmacological implications of estrogen receptor action. *Am. J. Pharmacogenomics* **4**, 19–28
- Montano, M. M., Jaiswal, A. K. and Katzenellenbogen, B. S. (1998) Transcriptional regulation of the human quinone reductase gene by antiestrogen-liganded estrogen receptor- α and estrogen receptor- β . *J. Biol. Chem.* **273**, 25443–25449
- Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J. et al. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl. Cancer Inst.* **90**, 1371–1388
- Veronesi, U., Maisonneuve, P., Rotmensz, N., Costa, A., Sacchini, V., Travaglini, R., D'Aiuto, G., Lovison, F., Gucciardo, G., Muraca, M. G. et al. (2003) Italian randomized trial among women with hysterectomy: tamoxifen and hormone-dependent breast cancer in high-risk women. *J. Natl. Cancer Inst.* **95**, 160–165
- Caulley, J. A., Norton, L., Lippman, M. E., Eckert, S., Krueger, K. A., Purdie, D. W., Farrerons, J., Karasik, A., Mellstrom, D., Ng, K. W. et al. (2001) Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. *Breast Cancer Res. Treat.* **65**, 125–134
- Bianco, N. R., Perry, G., Smith, M. A., Templeton, D. J. and Montano, M. M. (2003) Functional implications of antiestrogen induction of quinone reductase: inhibition of estrogen-induced DNA damage. *Mol. Endocrinol.* **17**, 1344–1355
- Castagnetta, L. A., Granata, O. M., Traina, A., Ravazzolo, B., Amoroso, M., Miele, M., Bellavia, V., Agostara, B. and Carruba, G. (2002) Tissue content of hydroxyestrogens in relation to survival of breast cancer patients. *Clin. Cancer Res.* **8**, 3146–3155
- Liehr, J. G. (2000) Is estradiol a genotoxic mutagenic carcinogen? *Endocrinol. Rev.* **21**, 40–54
- Bhat, H. K., Calaf, G., Hei, T. K., Loya, T. and Vadgama, J. V. (2003) Critical role of oxidative stress in estrogen-induced carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 3913–3918
- Hiraku, Y., Yamashita, N., Nishiguchi, M. and Kawanishi, S. (2001) Catechol estrogens induce oxidative DNA damage and estradiol enhances cell proliferation. *Int. J. Cancer* **92**, 333–337
- Roy, D. and Liehr, J. G. (1988) Temporary decrease in renal quinone reductase activity induced by chronic administration of estradiol to male Syrian hamsters. Increased superoxide formation by redox cycling of estrogen. *J. Biol. Chem.* **263**, 3646–3651
- Shen, L., Pisha, E., Huang, Z., Pezzuto, J. M., Krol, E., Alam, Z., van Breemen, R. B. and Bolton, J. L. (1997) Bioreductive activation of catechol estrogen-ortho-quinones: aromatization of the B ring in 4-hydroxyequilenin markedly alters quinoid formation and reactivity. *Carcinogenesis* **18**, 1093–1101
- Ross, J. A. and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **22**, 19–34
- This, P., De La Rochefordiere, A., Clough, K., Fourquet, A. and Magdelenat, H. (2001) Phytoestrogens after breast cancer. *Endocr. Relat. Cancer* **8**, 129–134
- Mizunuma, H., Kanazawa, K., Ogura, S., Otsuka, S. and Nagai, H. (2002) Anticarcinogenic effects of isoflavones may be mediated by genistein in mouse mammary tumor virus-induced breast cancer. *Oncology* **62**, 78–84
- Whitten, P. L. and Patisaul, H. B. (2001) Cross-species and interassay comparisons of phytoestrogen action. *Environ. Health Perspect.* **109** (Suppl. 1), 5–20
- Peeters, P. H., Keinan-Boker, L., van der Schouw, Y. T. and Grobbee, D. E. (2003) Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. *Breast Cancer Res. Treat.* **77**, 171–183
- An, J., Tzagarakis-Foster, C., Scharschmidt, T. C., Lomri, N. and Leitman, D. C. (2001) Estrogen receptor β -selective transcriptional activity and recruitment of coregulators by phytoestrogens. *J. Biol. Chem.* **276**, 17808–17814
- Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B. and Gustafsson, J. A. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* **139**, 4252–4263
- Morito, K., Aomori, T., Hirose, T., Kinjo, J., Hasegawa, J., Ogawa, S., Inoue, S., Muramatsu, M. and Masamune, Y. (2002) Interaction of phytoestrogens with estrogen receptors α and β (II). *Biol. Pharm. Bull.* **25**, 48–52
- Morito, K., Hirose, T., Kinjo, J., Hirakawa, T., Okawa, M., Nohara, T., Ogawa, S., Inoue, S., Muramatsu, M. and Masamune, Y. (2001) Interaction of phytoestrogens with estrogen receptors α and β . *Biol. Pharm. Bull.* **24**, 351–356
- Wang, W., Liu, L. Q., Higuchi, C. M. and Chen, H. (1998) Induction of NADPH:quinone reductase by dietary phytoestrogens in colonic Colo205 cells. *Biochem. Pharmacol.* **56**, 189–195
- Yang, S. H., Kim, J. S., Oh, T. J., Kim, M. S., Lee, S. W., Woo, S. K., Cho, H. S., Choi, Y. H., Kim, Y. H., Rha, S. Y. et al. (2003) Genome-scale analysis of resveratrol-induced gene expression profile in human ovarian cancer cells using a cDNA microarray. *Int. J. Oncol.* **22**, 741–750
- Heo, Y. H., Kim, S., Park, J. E., Jeong, L. S. and Lee, S. K. (2001) Induction of quinone reductase activity by stilbene analogs in mouse Hepa 1c1c7 cells. *Arch. Pharm. Res.* **24**, 597–600
- Montano, M. M. and Katzenellenbogen, B. S. (1997) The quinone reductase gene: a unique estrogen receptor-regulated gene that is activated by antiestrogens. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2581–2586
- Montano, M. M., Ekena, K., Krueger, K. D., Keller, A. L. and Katzenellenbogen, B. S. (1996) Human estrogen receptor ligand activity inversion mutants: receptors that interpret antiestrogens as estrogens and estrogens as antiestrogens and discriminate among different antiestrogens. *Mol. Endocrinol.* **10**, 230–242
- Paulus, W., Baur, I., Boyce, F. M., Breakfield, X. O. and Reeves, S. A. (1996) Self-contained, tetracycline regulated retroviral vector system for gene delivery to mammalian cells. *J. Virol.* **70**, 62–67
- Girdler, F., Browell, D. A., Cunliffe, W. J., Shenton, B. K., Hemming, J. D., Scorer, P., Young, J. R. and Brotherick, I. (2001) Use of the monoclonal antibody DAKO-ER β (8D5-8D1) to measure oestrogen receptor beta in breast cancer cells. *Cytometry* **45**, 65–72

- 31 Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C. and Klinge, C. M. (2000) Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β . *Endocrinology* **141**, 3657–3667
- 32 Montano, M. M., Wittmann, B. M. and Bianco, N. R. (2000) Identification and characterization of a novel factor that regulates quinone reductase gene transcriptional activity. *J. Biol. Chem.* **275**, 34306–34313
- 33 Fuqua, S. A., Schiff, R., Parra, I., Friedrichs, W. E., Su, J. L., McKee, D. D., Slentz-Kesler, K., Moore, L. B., Willson, T. M. and Moore, J. T. (1999) Expression of wild-type estrogen receptor beta and variant isoforms in human breast cancer. *Cancer Res.* **59**, 5425–5428
- 34 Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A. and Brown, M. (2000) Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell (Cambridge, Mass.)* **103**, 843–852
- 35 Dhakshinamoorthy, S., Long, D. J. and Jaiswal, A. K. (2000) Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens. *Curr. Top. Cell Regul.* **36**, 201–216
- 36 Nioi, P., McMahon, M., Itoh, K., Yamamoto, M. and Hayes, J. D. (2003) Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. *Biochem. J.* **374**, 337–348
- 37 Murata, M., Midorikawa, K., Koh, M., Umezawa, K. and Kawanishi, S. (2004) Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs. *Biochem. J.* **43**, 2569–2577
- 38 Nguyen, T., Yang, C. S. and Pickett, C. B. (2004) The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radical Biol. Med.* **37**, 433–441
- 39 Hayashi, S., Hajiro-Nakanishi, K., Makino, Y., Eguchi, H., Yodoi, J. and Tanaka, H. (1997) Functional modulation of estrogen receptor by redox state with reference to thioredoxin as a mediator. *Nucleic Acid Res.* **25**, 4035–4040
- 40 Maubach, J., Bracke, M. E., Heyerick, A., Depypere, H. T., Serreyn, R. F., Mareel, M. M. and De Keukeleire, D. (2003) Quantitation of soy-derived phytoestrogens in human breast tissue and biological fluids by high-performance liquid chromatography. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* **784**, 137–144
- 41 Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engstrom, O., Ljunggren, J., Gustafsson, J. A. and Carlquist, M. (1999) Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. *EMBO J.* **18**, 4608–4618
- 42 Wei, H., Cai, Q. and Rahn, R. O. (1996) Inhibition of UV light- and Fenton reaction-induced oxidative DNA damage by the soybean isoflavone genistein. *Carcinogenesis* **17**, 73–77
- 43 Wei, H., Zhang, X., Wang, Y. and Lebwohl, M. (2002) Inhibition of ultraviolet light-induced oxidative events in the skin and internal organs of hairless mice by isoflavone genistein. *Cancer Lett.* **185**, 21–29
- 44 Seike, N., Wanibuchi, H., Morimura, K., Wei, M., Nishikawa, T., Hirata, K., Yoshikawa, J. and Fukushima, S. (2003) Enhancement of lung carcinogenesis by nonylphenol and genistein in a F344 rat multiorgan carcinogenesis model. *Cancer Lett.* **192**, 25–36
- 45 Peterson, T. G., Coward, L., Kirk, M., Falany, C. N. and Barnes, S. (1996) The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. *Carcinogenesis* **17**, 1861–1869
- 46 Kulling, S. E., Lehmann, L. and Metzler, M. (2002) Oxidative metabolism and genotoxic potential of major isoflavone phytoestrogens. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* **777**, 211–218
- 47 Kulling, S. E., Honig, D. M. and Metzler, M. (2001) Oxidative metabolism of the soy isoflavones daidzein and genistein in humans *in vitro* and *in vivo*. *J. Agric. Food Chem.* **49**, 3024–3033
- 48 Magee, P. J. and Rowland, R. I. (2004) Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. *Br. J. Nutr.* **91**, 513–531

Received 7 June 2004/16 August 2004; accepted 30 September 2004

Published as BJ Immediate Publication 30 September 2004, DOI 10.1042/BJ20040959