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

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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 ERβ (oestrogen receptor β) transactivation. Such QR induction appears to protect breast cells against oestrogen-induced oxidative DNA damage, most likely by reducing reactive oestrogen metabolites termed catecholestrogens back to the hydroxy-catecholestrogens 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 ERβ transactivation at the electrophile response element of the QR gene promoter. By chromatin immunoprecipitation analysis, we show binding of ERα and ERβ to the QR promoter, with increased ERβ 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 ERβ or QR. Our results with resveratrol are consistent with our hypothesis that the protective ability of resveratrol is partially dependent on the presence of ERβ 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 observed that QR may be transcriptionally activated by anti-oestrogen-bound ER, preferentially the ERβ 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-catecholestrogens, and further oxidized to the semiquinone and quinone form [9,10]. This metabolism is potentially harmful, given that the quinone-catecholestrogen 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-catecholestrogens back to the hydroxy-catecholestrogens, 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 catecholestrogens, as well as direct reaction of the quinone-catecholestrogens 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; ERα, ERβ antisense; Gen, genistein; NAC, N-acetylcysteine; 8-OHdG, 8-hydroxydeoxyguanosine; QR, quinone reductase; QRα, QRβ antisense; Res, resveratrol; SERM, selective oestrogen receptor modulator; TBHQ, t-butylhydroquinone; TK, thymidine kinase.

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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 ERβ [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-ERβ 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 ERβ transactivation. We show that ERα and ERβ bind to the QR promoter in vivo, with increased ERβ 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 ERβ, as knock-down of QR and ERβ 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 μg of the EpRE–tk–luciferase reporter constructs, 100 ng of pCMV5-ER expression vector and 0.15 μg 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 μg) 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 μg) 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). ERα 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 ERβ 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 μM) 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, resuspended in PBS, centri fuged and washed twice for 10 min each with the following buffers at 4°C: 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 μl of a Protein A/salmon sperm DNA/rRNA mixture (1 ml of Protein A slurry, 24 μl of 10 mg/ml salmon sperm DNA, 24 μl of 10 mg/ml tRNA). The supernatant was incubated overnight at 4°C with 5 μg of antibody. Antibodies for ERα, ERβ, 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 μl of the Protein A/salmon sperm DNA/rRNA 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 × 250 μl 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 μl 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 μl 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′-CATGCACCAGGGAGTGTTGTG-3′; for the tetO, QR(−393/−417) 5′-GCACGAAATGGAGCAGAAAAAGAG-3′; and for the transactivator protein tTA, causing it to dissociate from the tETO minimal CMV promoter, 5′-CGGCGATTGGATTAATGATGG-3′. Gene expression is inhibited by tetracycline, which binds the transactivator protein tTA, and the regulator unit, encoding the tTA protein (the tetracycline regulator 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, ERα or ERβ 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.

MCF7 cells were transfected with a pBPSTR1 plasmid containing antisense QR or ERβ, or pBPSTR1 containing sense ERα or ERβ. 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 μg/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, ERα or ERβ 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).

Immunocytochemistry for 8-OHdG in breast cells
This procedure has been described in detail previously [8]. Cells grown on coverslips were fixed in methanol (methanol/chloroform/acidic 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 μg/ml in PBS, pH 7.4, for 15 min at room temperature). To detect oxidized nucleotides, 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.
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 revealed isoform-specific induction of EpRE activity by the phytoestrogens. The induction was significantly greater when ERβ was over-expressed in the absence of ligand (Figure 3).

**Phytoestrogen regulation of QR gene transcriptional activity by ERβ**

Phytoestrogens, including Bio A and Gen used in the present study, bind preferentially to the ERβ isoform [22]. Res, however, binds to ERα and ERβ with similar affinity [31]. To determine which isoform is mediating the phytoestrogen induction of QR, we co-transfected an expression plasmid for ERα or ERβ along with the reporter gene EpRE-tk-pGL3. The cells were then transfected with the indicated concentrations of Bio A, Gen, Res or the positive control TBHQ (10 μM). The induction was measured, and fold activation over vehicle treatment of cells not transfected with ERα or ERβ was displayed as an average ± S.E.M. for three independent experiments. *P < 0.05 and **P < 0.001 when compared with ERα over-expressing cells treated with vehicle alone as determined by the Student’s t-test.

**Figure 3** EpRE-mediated transcriptional activation by phytoestrogens

MDA-MB-231 breast cancer cells were co-transfected with control expression vector (no ERα or ERβ cDNA) or an expression vector for ERα or ERβ 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 μM). 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 ERα or ERβ is displayed as the means ± S.E.M. for three independent experiments. *P < 0.05 and **P < 0.001 when compared with ERα over-expressing cells treated with vehicle alone as determined by ANOVA.

**Figure 4** ERβ is the isoform critical for EpRE activation

MDA-MB-231 breast cancer cells were co-transfected with control expression vector (no ERα or ERβ cDNA) or an expression vector for ERα or ERβ along with the reporter gene EpRE-tk-pGL3 and treated for 48 h with the indicated concentrations of Bio A, Gen, Res or the positive control TBHQ (10 μM). 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 ERα or ERβ is displayed as an average ± S.E.M. for three independent experiments. *P < 0.05 and **P < 0.001 when compared with control cells or cells expressing ERα with the same treatment as determined by ANOVA.
breast cancer cells were transfected with an expression vector for ERβ A shown in (Gen and NAC (G cells treated for 45 min with vehicle). Antibody against MafK or ERβ (row 4). (EpRE (lanes 7–8). Input chromatin (2%) was also analysed by PCR for a comparison control antibody (row 3). Bound QR promoter-containing chromatin was detected by PCR analysis using no primary antibody (lane 6). As a positive control, chromatin extracts were incubated with Nrf2 with vehicle (lane 4), and incubated with antibody against ERβ (lane 2). In the presence of TBHQ, a b-zip (leucine zipper) transcription factor that positively regulates EpRE-mediated transactivation of the QR gene. We used Nrf2 in the present study as a positive control to show the assay working properly. In addition, 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. However, we observed no change in MafK binding, relative to control, in the presence of resveratrol, which we have shown to induce increased ERβ 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 ERβ 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.

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. ERβ showed no change in MafK binding, relative to control, in the presence of resveratrol, which we have shown to induce increased ERβ binding (Figure 5B, lanes 4 and 8). However, MafK is also seen with anti-oestrogen regulation [4].

ERβ binding to the EpRE region of the QR promoter

ERα and ERβ 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 ERα or ERβ, 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 µM) 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 ERα and ERβ (Figure 5A). However, in 3 out of 4 independent experiments, there was a consistent increase in ERβ binding in the presence of Res when compared with vehicle alone. ERα 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. We used Nrf2 in the present study as a positive control to show the assay 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 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. However, we observed no change in MafK binding, relative to control, in the presence of resveratrol, which we have shown to induce increased ERβ 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 ERβ 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.

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

gene EpRE-α, p65, and treated for 48 h with the vehicle, 10 µg 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 ± S.E.M. for three independent experiments.
inducing Nrf2 nuclear translocation, EpRE binding and perhaps recruitment of ERβ. 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 ERβ 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 may 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 the same ability. To test this, MCF7 cells were treated with oestrogen (10 nM) with or without the phytoestrogens Bio A, Gen or Res at 10 μM for 24 h, and immunostained for 8-OHdG. 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 ERβ 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 μM, 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 ERβ and QR levels

(A) MCF7 cells were transiently infected with ERβ 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 µM) 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 QRAS 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 µM) 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, ERα retroviruses or ERβ retroviruses. The cells were then treated with vehicle (control) or E2 (10 nM) with or without Res (1 µM) 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 ± S.E.M. of three adjacent fields from three or more separate experiments. Lower panels: Western blot analyses for ERα or ERβ in MCF10A cells infected with control, ERα or ERβ retroviruses. NSB is a non-specific band observed in the Western blot.

At least in the case of Res, this protection relies on ERβ and QR, as a reduction in the levels of these two proteins diminishes the protective effect. These results mimic those seen with the anti-oestrogen tamoxifen [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 ERβ 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 co-activator-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 ERβ ligand-binding domain in the presence of Gen adopts a conformation very similar to that of anti-oestrogen-bound ERβ [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 QRAS-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 ERβ suggests that ERβ 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 ERβ 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 ERβ 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 μM, 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-κB, and (7) induction of 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.

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Phytoestrogens induce quinone reductase


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