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Soy isoflavones increase quinone reductase in hepa-1c1c7 cells via estrogen receptor beta and nuclear factor erythroid 2-related factor 2 binding to the antioxidant response element☆

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Abstract

Soy protein and isoflavones (genistein and daidzein) have been demonstrated to increase quinone reductase (QR) activity, protein, and mRNA in animal and cell culture models. However, their mechanism of action has not been completely characterized. Additionally, it has not been determined if equol, a daidzein metabolite, can modulate QR activity and expression. Estrogen receptor beta (ERβ) is thought to be involved in stimulating QR gene transcription by anti-estrogens and phytoestrogens, along with nuclear factor erythroid 2-related factor 2 (Nrf2). This study tested the hypothesis that genistein, daidzein and equol increase quinone reductase activity, protein and mRNA via ERβ and Nrf2 binding to the QR antioxidant response element (ARE). QR expression and activity were determined using TaqMan polymerase chain reaction, protein immunoblots and activity assays. Molecular events were investigated using luciferase reporter gene assays and chromatin immunoprecipitation (ChIP). Hepa-1c1c7 cells were treated with control [0.1% (v:v) dimethyl sulfoxide (DMSO)]; 1 μmol/L β-naphthoflavone (positive control); 5 μmol/L resveratrol (ChIP positive control for ERβ binding) and 1, 5 and 25 μmol/L genistein, daidzein or equol. Treatment durations were 1 h (ChIP), 24 h (mRNA and luciferase assays) and 24 and 48 h (protein and activity). Genistein, daidzein and equol increased QR activity, protein and mRNA, with daidzein and equol having more of an impact at physiologic concentrations (1 and 5 μmol/L) compared to genistein. Furthermore, the study results demonstrate that genistein, daidzein and equol interact with the QR ARE and that daidzein and equol act via both ERβ and Nrf2 binding strongly to the QR ARE.

Keywords: Isoflavone; Quinone reductase; Phase II enzyme; Antioxidant response element; soy; equol

1. Introduction

Quinone reductase or NQO1 (QR) [NAD(P)H:quinone oxidoreductase; DT-diaphorase] is a phase II xenobiotic metabolizing enzyme which catalyzes the obligatory two-electron reduction of a wide range of endogenous and exogenous quinones and quinoid compounds [1] and protects cells against reactive oxygen species generated by quinones and related electrophilic compounds [2]. Induction of QR activity has been suggested to assist in chemoprevention against cancer and chemical toxicity by natural or synthetic compounds [3,4]. Conversely, QR polymorphisms or experimental disruption of the gene is associated with increased susceptibility to carcinogenesis and toxicity [5,6].

QR is a cytosolic enzyme expressed in a variety of mammalian tissues and cell types and is highly induced by arylhydrocarbon receptor (AhR) agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dixoin, polycyclic aromatic hydrocarbons and by phenolic antioxidants such as 2-*t*-butylbenzene-1,4-diol [7]. Dietary flavonoids from a variety of fruits, vegetables and beverages have also been shown to be potent inducers of this enzyme [8,9]. Soy phytochemicals can modulate both phase I and II enzymes in the xenobiotic metabolizing system [10–13]. In vivo and in vitro experiments have demonstrated that soy and soy phytochemicals specifically induce QR activity [14–16].

Expression of phase II enzymes is regulated in part through activation and translocation of the redox-sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) into the nucleus where it forms a heterodimer with other transcription factors and binds to the antioxidant response element (ARE) of genes such as QR, initiating transcription [17–20]. The molecular mechanism whereby isoflavones regulate hepatic QR transcription is not known with certainty. They may play a role in modulating kinase pathways affecting AhR or Nrf2, or as a factor which disrupts Nrf2-Keap1 association and therefore increasing Nrf2 translocation [1,20–23]. Another possible mode of action recently reported in breast cancer cells is for phytoestrogens to act through estrogen receptor beta (ERβ) transactivation at the ARE of the QR enhancer region, similar to the actions of anti-estrogens [24].

The aim of the current study was to examine hepatic QR enzyme levels and nuclear events involved in gene regulation in response to soy isoflavone aglycones genistein, daidzein and the daidzein metabolite equol. This is of importance for extending knowledge

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about potential roles of isoflavones in cancer prevention. Isoflavones circulate in physiologic form predominately as conjugates following biotransformation in intestinal epithelium and liver, while the intracellular forms are not known with certainty. In this in vitro study, we used commercially available aglycones at physiologic concentrations (1 and 5 μmol/L) that represent the low and high values of human serum genistein concentrations after the consumption of a soy rich meal [25–29]. Furthermore, the 24-h time points (used to measure luciferase activity, and QR mRNA, protein and activity) cover the human peak serum genistein concentrations of ~6.5 h followed by a half-life of ~10 h [30]. We tested the hypothesis that soy isoflavones increase QR via ERβ and Nrf2 binding to the QR ARE in mouse Hepa-1c1c7 cells treated with soy isoflavones. The mechanisms of QR regulation were investigated by using luciferase reporter gene assays, chromatin immunoprecipitation techniques, polymerase chain reaction (PCR) analysis, Western immunoblots and enzyme activity assays.

2. Methods and materials

2.1. Chemicals and reagents

Genistein, daidzein, equol, and resveratrol (ChIP positive control for ERβ binding) were purchased from Indofine Chemical Company. β-naphthoflavone was purchased from Sigma-Aldrich and used as a positive control for QR activity, Western blot, TaqMan PCR, and luciferase reporter gene assays. Cell culture medium and reagents were purchased from Invitrogen. Quinone reductase (QR) and actin antibodies were purchased from Santa Cruz Biotechnology. Western blot buffers and reagents were purchased from Bio-Rad Laboratories and Santa Cruz Biotechnology. QR TaqMan reagents, primers, and probe were purchased from Applied Biosystems. Estrogen receptor beta (ERβ) and nuclear factor erythroid 2-related factor 2 (Nrf2) antibodies used in the chromatin immunoprecipitation (ChIP) assays were purchased from Santa Cruz Biotechnology. Unless noted otherwise, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

2.2. Cell culture and treatments

Hepa-1c1c7 cells, purchased from American Type Culture Collection, were grown in 75-cm^2 flasks in an incubator at 37°C and 5% CO_2, with Minimum Essential Medium, 10% charcoal-treated fetal bovine serum (qualified), and 1% penicillin/streptomycin (all purchased from Invitrogen). The medium was changed every 3 d, and when confluency was observed the cells were split using trypsin. Cell culture experiments were done in triplicate and repeated three times.

Hepa-1c1c7 cells were exposed to control [0.1% (v:v) DMSO]; 1 μmol/L β-naphthoflavone (positive control); 5 μmol/L resveratrol (ChIP positive control) or 1, 5 and 25 μmol/L genistein, daidzein or equol (all aglycones). The treatment durations were for 1 h (ChIP), 24 h (mRNA and luciferase assays) and 24 and 48 h (QR activity and protein). QR activity, protein, mRNA, QR ARE luciferase activity and ChIP were measured at the appropriate time points to determine if genistein, daidzein and equol increase QR via ERβ and Nrf2 binding to the QR ARE. The concentrations used in this study (1–25 μmol/L) were less than the concentrations (5–40 μmol/L) of genistein and daidzein treatment to Hepa-1c1c7 cells reported previously without evidence of toxic effects or disruption of cell viability [15].

2.3. Quinone reductase activity assay

The QR activity assay was based on the Prochaska method [31]. In summary, Hepa-1c1c7 cells were seeded in 96-well plates at a density of 10^4 cells per well and grown in an incubator at 37°C and 5% CO_2 for 24 h. The medium was removed and replaced with fresh medium containing the appropriate phytochemical concentrations. After the cells were treated for 24 or 48 h, the medium was removed and replaced with 50 μl digitonin lysing solution for 10 min at 37°C and 5% CO_2. The 96-well plates were transferred to an orbital shaker for 10 min at 100 rpm and 25°C. Protein was measured using the Bio-Rad protein assay (Bio-Rad Laboratories), based on the Bradford method. Cytosolic QR 1 activity was measured in 96-well plates by following the conversion of glucose 6-phosphate+NADP to 6-phospho-gluconate+NADPH by glucose 6-phosphate dehydrogenase. The reactions that followed were NADPH+menadione to menadiol+NADP by QR 1 and the linked reduction of thiazolyl blue tetrazolium bromide. This reduction was measured spectrophotometrically at 600 nm for 5 min. The fold of control increases were obtained by dividing the treated values by the control values.

2.4. Protein extraction and western immunoblot analysis

Hepa-1c1c7 cells were seeded in 100 mm culture dishes at a density of 10^6 cells per dish and grown in an incubator at 37°C and 5% CO_2 until ~90% confluent. The medium was replaced with fresh medium containing the appropriate phytochemical concentrations, followed by treatment of the cells for 24 and 48 h. The cells were washed with ice-cold phosphate-buffered saline (PBS) and protease inhibitor cocktail (Sigma), followed by the addition of 100 μl cell lysis buffer [20 mmol/L HEPES, 1 mmol/L EDTA, 10% (v:v) glycerol, 1% (v:v) Triton X-100 and protease inhibitor cocktail] [32]. The cells were collected with a rubber policeman and dispensed into 1.5-ml tubes on ice. The lysates were incubated on ice for 1 h with intermittent vortexing every 10 min, followed by centrifugation at 12,000×*g* for 10 min at 4°C. The cytosolic supernatants were collected and stored at −80°C until further analysis.

Western blots were performed on 24 and 48 h treatment groups. Protein was measured using the Bio-Rad assay. Protein (50 μg cytosolic protein/well) was loaded and separated on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories). The separated proteins were blotted onto PVDF membranes and incubated with blocking reagent (TBS [Tris Buffered Saline, with 5% milk and 0.05% Tween 20] Blotto A, Santa Cruz Biotechnology) overnight at 4°C. The proteins were probed with antibodies directed against QR or actin at a concentration of 1:200 for 1 h. The membranes were washed with TBS [10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% (v:v) Tween 20, pH 8.0]. The membranes were subsequently exposed to horseradish peroxidase-linked donkey anti-goat IgG antibodies (1:5000) for 1 h and washed with TBS, followed by a TBS wash without Tween 20. Western blot signals were detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Santa Cruz Biotechnology). Immunoquantitation was determined using the ChemiDoc XRS densitometric scanning imager (Bio-Rad Laboratories).

2.5. RNA extraction and real-time PCR (TaqMan) analysis

Hepa-1c1c7 cells were seeded in six-well plates at a density of 10^6 cells per well and grown in an incubator at 37°C and 5% CO_2 for 24 h. The medium was replaced with fresh medium containing the appropriate phytochemical concentrations, followed by a 24-h treatment period. The cells were lysed, and the mRNA was isolated following the manufacturer's instructions for the RNeasy Plus mini kit (Qiagen). Single-stranded cDNA was synthesized using oligo (dT) primers and SuperScript II Reverse Transcriptase (Invitrogen) by following the manufacturer's protocol. TaqMan PCR was utilized to quantify the amount of QR mRNA (National Center for Biotechnology Information [NCBI] Reference Sequence NM 008706.5) for the various treatments using primers and probe from Applied Biosystems. The cycle parameters were as follows: first cycle at 50°C for 2 min, followed by 95°C for 10 min, and 40 cycles at 95°C for 15 s, followed by 60°C for 1 min. The comparative Ct method was used to determine the fold induction for each treatment, with actin used as the internal standard.

2.6. Plasmids

The 5′-upstream region of *nqo1* cloned into the pGL3-Basic (Promega) luciferase reporter vector to create -1016/*nqo5′-luc*, was generously provided by Dr. John D. Hayes (Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Scotland, UK). The plasmid containing the luciferase construct was propagated in competent Escherichia coli cells (Genesee Scientific). Plasmid DNA was prepared using the QIAprep Spin Miniprep kit (Qiagen). The pRL-TK *Renilla* reporter vector (Promega) was used as an internal control.

2.7. DNA transfection and luciferase reporter gene assay

At ~90% confluency, Hepa-1c1c7 cells were cotransfected with 0.1 μg of −1016/*nqo5′-luc* plasmid and 0.1 μg of pRL-TK *Renilla* reporter vector using Lipofectamine 2000 (Invitrogen), according to the manufacturer's guidelines. After 24 h of transfection, the cells were treated with control [0.1% (v:v) DMSO]; 1 μmol/L β-naphthoflavone (positive control) or 1 and 5 μmol/L genistein, daidzein, equol or resveratrol for 24 h. Following treatment, firefly (−1016/*nqo5′-luc*) and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega) and a Wallac 1420 luminometer (PerkinElmer). Background luminescence was subtracted using nontransfected cells, and luciferase activities were normalized to the internal control *Renilla* luminescence.

2.8. Chromatin immunoprecipitation assay

Hepa-1c1c7 cells were seeded in three 100 mm culture dishes at a density of 10^6 cells per dish for each treatment and grown in an incubator at 37°C and 5% CO_2 until confluent. The medium was replaced with fresh medium containing the appropriate phytochemical concentrations. The cells were treated with control [0.1% (v:v) DMSO]; 1 μmol/L β-naphthoflavone or 5 μmol/L genistein, daidzein, equol or resveratrol for 1 h. The cells were processed using the ChIP-IT Enzymatic kit (Active Motif). Briefly, the procedure was initiated by cross-linking the proteins to DNA via 1% formaldehyde to the culture dishes for 10 min on a rocking platform at room temperature. The cross-linking was stopped with the addition of glycine solution. The cells were scraped with a rubber policeman using 2 ml of scraping solution (PBS supplemented with 0.5 mmol/L phenylmethanesulfonyl fluoride [PMSF]) and pooled from three plates, followed by centrifugation for 10 min at

720×g. The protocol was continued according to the manufacturer's instructions. During the ChIP procedure, ERβ and Nrf2 antibodies (2 μg each) were added to the pre-cleared chromatin in 0.65-ml siliconized tubes and incubated overnight on a rotator at 4°C. The QR ARE DNA (obtained from the ChIP procedure) was analyzed using PCR with previously described primers [33] and Platinum Taq High Fidelity (Invitrogen). The cycle parameters were as follows: first cycle at 94°C for 2 min, 35 cycles at 94°C for 20 s, 55°C for 30 s, 68°C for 30 s and a final extension at 68°C for 7 min. PCR products were resolved on a 1% agarose gel containing ethidium bromide, and visualized under UV illumination using the Bio-Rad imaging system.

2.9. Statistical analysis

Data are expressed as means±S.D. All data were analyzed using SigmaStat version 3.5 for Windows (Systat Software). Data were analyzed using analysis of variance (ANOVA), followed by Student-Newman-Keuls post hoc test that compared treatments to control. An α level of .05 was used to determine significance. The graphs for the figures were prepared using GraphPad Prism version 5.02 for Windows (GraphPad Software).

3. Results

3.1. Daidzein and equol increase quinone reductase activity at physiologic concentrations

Enzymatic activity assays demonstrated that QR is modulated by genistein, daidzein and equol. Treatment of the Hepa-1c1c7 cells with daidzein and equol at 1, 5 and 25 μmol/L for 24 and 48 h significantly increased QR specific activity at each concentration and time point (Fig. 1A and B). Daidzein resulted in 1.21–1.42-fold of control increased QR activity, and equol treatment yielded 1.28–1.65-fold of control increases compared to control [0.1% (v:v) DMSO]. In contrast, 1 μmol/L genistein treatment did not significantly increase QR activity at either time point. Treatment with the higher concentrations of 5 and 25 μmol/L resulted in QR activity which was increased 1.28–1.63-fold of control compared to control. The cells treated with 1 μmol/L of the positive control β-naphthoflavone (NF) strongly increased QR activity after 24 and 48 h (1.91 and 2.0 fold of control, respectively).

3.2. Daidzein and equol increase mRNA at physiologic concentrations

QR mRNA was measured to determine if the soy isoflavones induce QR at the gene transcription level. Daidzein and equol both significantly increased QR mRNA following 24 h treatment of Hepa-1c1c7 cells with isoflavone concentrations of 1, 5 and 25 μmol/L (Fig. 1C). The mRNA levels were induced by 1.8–3.7-fold of control compared to controls [0.1% (v:v) DMSO]. Positive control NF also increased mRNA at its 1 μmol/L (9.17-fold of control) concentration. Genistein increased QR mRNA at 25 μmol/L (3.5-fold of control) but not following treatment at the lower concentrations.

3.3. Genistein, daidzein and equol increase quinone reductase protein at 48 h

Western immunoblot assays were performed to support the increases in QR activity and mRNA following exposure to soy isoflavones. Hepa-1c1c7 cells treated with 1 μmol/L NF (positive

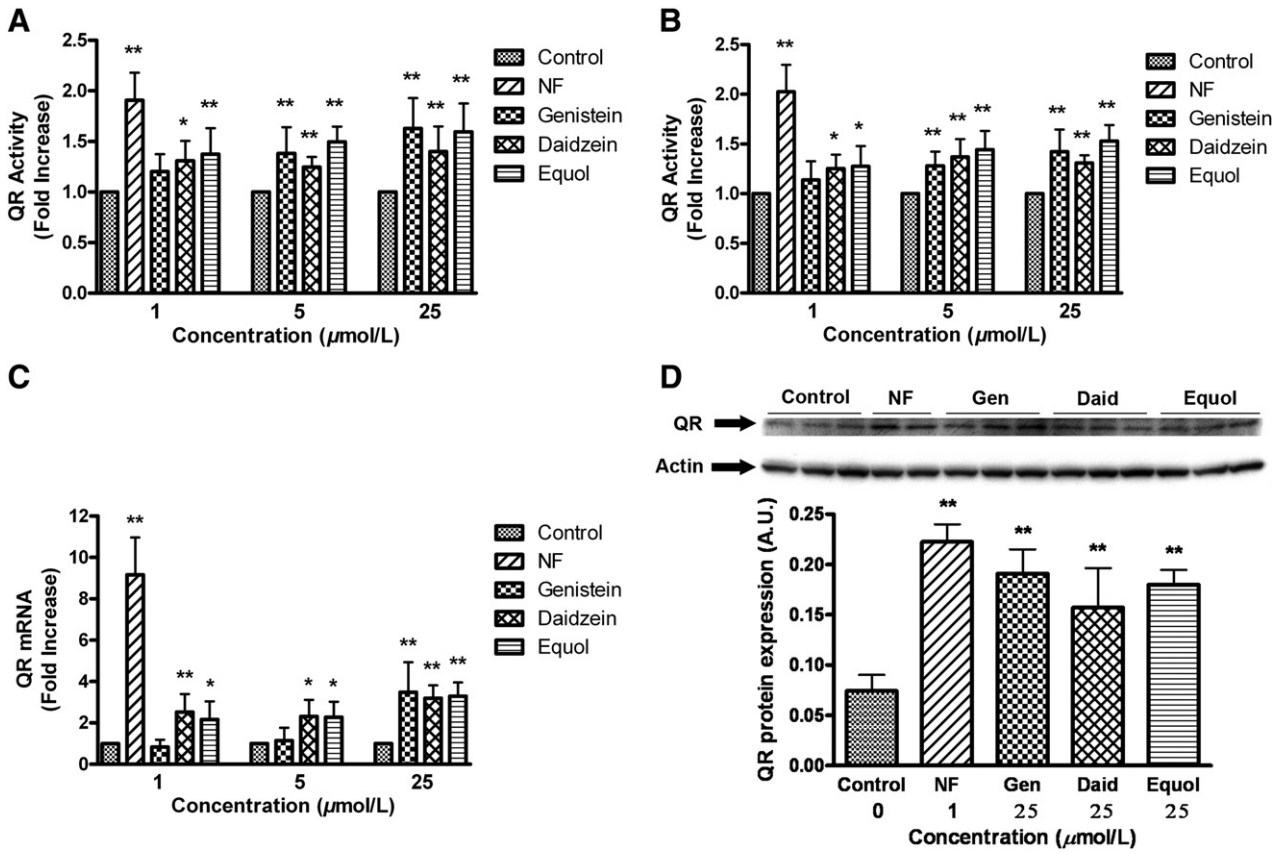

Fig. 1. Quinone reductase activity expressed as fold increases for Hepa-1c1c7 cells treated for 24 h (A) and 48 h (B). Real-time (TaqMan) PCR analysis (C) of the quinone reductase gene for Hepa-1c1c7 cells treated for 24 h and normalized to β-actin. Representative quinone reductase Western immunoblots (D) for Hepa-1c1c7 cells treated for 48 h and normalized to actin. For the enzyme activity, TaqMan, and Western immunoblot assays, Hepa-1c1c7 cells were treated with control [0.1% (v:v) DMSO], positive control 1 μmol/L β-naphthoflavone (NF), or 1, 5, and 25 μmol/L genistein (Gen), daidzein (Daid), or equol. Groups indicated by * ($P<.05$) or ** ($P<.001$) differ significantly compared to control. The results are expressed as means±S.D. from 3 separate experiments performed in triplicate.

control) or 25 μmol/L genistein, daidzein or equol for 48 h strongly increased QR protein ($P<.001$) compared to control [0.1% (v:v) DMSO] and normalized to actin (Fig. 1D). The less sensitive Western immunoblots (compared to the enzyme activity and TaqMan assays) did not result in strong changes in protein levels following 24 h treatment conditions, nor were there any significant changes at the lower concentrations (1 and 5 μmol/L) even after 48 h, despite induction of enzyme activity and mRNA at these concentrations and time points (data not shown). Cell viability as assessed by visual inspection of cell morphology and trypan blue exclusion indicated excellent cell viability with no evidence of toxicity at the treatment concentrations used.

3.4. Genistein, daidzein and equol increase luciferase activity in cells transiently transfected with the QR antioxidant response element

Physiologic concentrations (1 and 5 μmol/L) of genistein, daidzein and equol activate the QR antioxidant response element as demonstrated by Hepa-1c1c7 cells transiently transfected with the –1016/*nqo5′-luc* reporter plasmid. 24 h treatment of soy isoflavones genistein, daidzein, equol or positive controls NF and resveratrol resulted in significantly increased luciferase expression and activity for each treatment ($P<.001$) compared to DMSO control and normalized to *Renilla* luciferase activity (Fig. 2). The magnitude of change was similar for each of the isoflavones, ranging from 1.3- to 2.4-fold induction, with daidzein having the strongest effect.

3.5. Daidzein and equol strongly increase the binding of ERβ and Nrf2 to the QR antioxidant response element

ChIP assays were used to determine a mechanism for the increased -1016/*nqo5′-luc* activity in the transfected cells observed with genistein, daidzein and equol treatments. ERβ has been shown to bind to the QR ARE in MCF-7 cells with resveratrol treatment, but not genistein [24]; thus, we investigated if genistein, daidzein and equol might increase ERβ and Nrf2 binding to the QR ARE in Hepa-1c1c7 cells. The cells were treated for 1 h with control [0.1% (v:v) DMSO]; 1 μmol/L NF or 5 μmol/L genistein, daidzein, equol or resveratrol (positive control for ERβ binding). Daidzein, equol and resveratrol strongly increased the binding of ERβ to the QR antioxidant response element ($P<.001$) compared to control (Fig. 3). The increases ranged from 2.0–2.7-fold of control, with daidzein having the strongest effect. In contrast, genistein and NF had a lower impact on ERβ binding, with approximately 1.5 fold of control increases compared to control. All treatments increased the binding of the transcription factor Nrf2 to the QR ARE compared to control ($P<.001$). The magnitude of change ranged from 1.7 to 2.1-fold of control, with daidzein having the strongest effect.

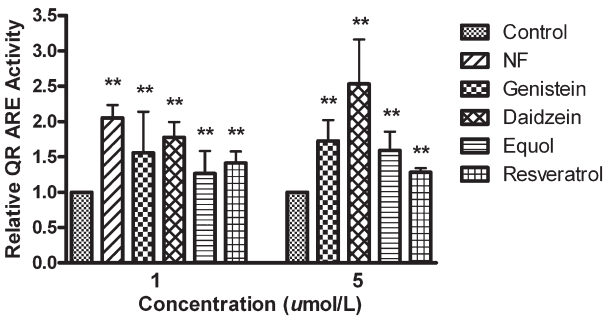

Fig. 2. Firefly luciferase activity for Hepa-1c1c7 cells transiently transfected with the quinone reductase antioxidant response element. The cells were treated with control [0.1% (v:v) DMSO], 1 μmol/L β-naphthoflavone (NF) (positive control), or 1 and 5 μmol/L genistein, daidzein, equol, or resveratrol for 24 h. Groups indicated by ** ($P<.001$) differ significantly compared to control. The results are expressed as means±S.D. from three separate experiments, and normalized to *Renilla* luciferase activity.

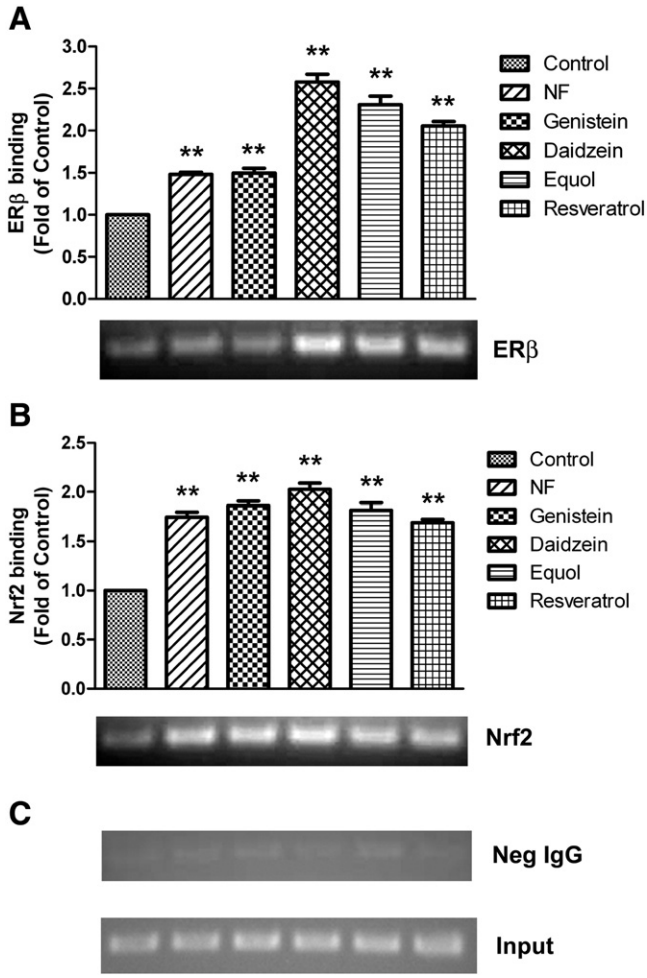

Fig. 3. Representative chromatin immunoprecipitation assays for ERβ (A) and Nrf2 (B) binding to the QR ARE in Hepa-1c1c7 cells. The cells were treated with control [0.1% (v:v) DMSO], 1 μmol/L β-NF or 5 μmol/L genistein, daidzein, equol or resveratrol (positive control for ERβ binding) for 1 h. ERβ and Nrf2 antibodies (2 μg each) were added to the pre-cleared chromatin. Negative IgG (2 μg) and input DNA (C) were used as controls. Primers specific to the mouse QR ARE were used for PCR analysis. Groups indicated by ** ($P<.001$) differ significantly compared to control. The results are expressed as means ±S.D. from three separate experiments.

4. Discussion

Dietary isoflavones mediate effects on biotransformation enzymes and ultimately on cancer prevention, but the underlying mechanisms of the aglycones and their physiologic conjugates have not been fully elucidated. This study demonstrates that soy isoflavone aglycones and the metabolite equol modulate quinone reductase (QR) at the molecular level by interacting with the QR antioxidant response element (ARE) and increasing the binding of estrogen receptor beta (ERβ) and nuclear factor erythroid 2-related factor 2 (Nrf2). Genistein, daidzein and equol increased QR activity, protein and mRNA under the experimental conditions employed in our work. Physiologic concentrations (1 and 5 μmol/L) of daidzein and equol treatments resulted in significant increases in QR mRNA and activity. The results of the transient transfections with −1016/*nqo5′-luc*

reporter plasmid are similar to findings with sulforaphane increasing −1016/*nqo5′-luc* activity in Hepa-1c1c7 cells [33] and of other phytochemicals such as epigallocatechin gallate (ECGC) [34]; thus, certain phytochemicals have the ability to increase ARE-luciferase reporter gene transactivation. To our knowledge, this is the first study to report genistein, daidzein and equol interact with the QR antioxidant response element.

The increases in QR activity observed in this study are similar to other studies using cell culture [15], mice [35], rats [14,36] and our previous study where hepatic QR activity was increased in male and female Swiss Webster mice fed 1500 mg/kg daidzein; yet, genistein did not impact hepatic QR activity compared to controls [37]. The more robust induction of QR activity by daidzein compared to genistein in this study differs from Wang et al. [38] who treated colonic cells with various concentrations of genistein and daidzein. An explanation for this difference might be different cell types and study design. We noted relatively larger increases in mRNA which were not reflected completely in activity. The observation of increases in QR protein and mRNA agree with Bianco et al. [24].

Equol has not been studied to determine its impact on QR activity, protein and mRNA in a cell culture model. Equol is produced from the intestinal bacterial metabolism of daidzein and might be responsible for some of the effects observed with daidzein in previous animal studies. Approximately 30–50% of humans are equol producers, whereas all rodents produce equol [39]. Therefore, this can account for some of the differing results seen in human [40], cell culture [15,38] and animal studies [36,39,41], regarding daidzein. Furthermore, approximately 50% of equol circulates in an unbound form, and both equol and genistein have similar high binding affinities to ERβ, making them both candidates for modulating gene expression via ERβ [39,42].

To further investigate the mechanism of QR modulation by soy isoflavones, ChIP was utilized to identify transcription factors involved in the increases in QR. The relative binding affinities (in comparison to estradiol) for genistein, daidzein and equol to ERβ are 18.13, 0.79 and 1.87, respectively; however, the transactivation activities are 84.76, 80.95, and 96.16 [43]. The transactivation characteristics make these isoflavones potent candidates for modulating gene transcription. In this study, equol and daidzein increased transcription of QR via both ERβ and Nrf2 strongly binding to the QR antioxidant response element. The ChIP results for ERβ are in agreement regarding resveratrol exposure but differ for genistein treatment observed in the study by Bianco et al. [24] where a different cell type (MCF-7 breast cancer cell) and treatment concentration (1 μmol/L) were used. In addition to liver and breast, ERβ is found in high concentrations in the prostate, epididymis, lung and hypothalamus [42]. Therefore, there is the potential for differential ERβ induction by isoflavones, reflecting tissue distribution and enrichment.

Possible explanations for the decreased impact of genistein (compared to daidzein and equol) on QR include: (1) genistein binding to ERα which precipitates Nrf2, inhibiting it from translocating into the nucleus and binding to the ARE, resulting in decreased transcription of the QR gene [20,44,45]; however, genistein did not decrease Nrf2 binding to the QR ARE in this study; (2) inducing oxidation of the cysteine residues in ERβ, which has been shown to inhibit ER-DNA interaction [46]; however, there are contradictory results in Nrf2 or ERβ binding in the presence of genistein or in combination with the antioxidant N-acetylcysteine (NAC) [24]; or (3) kinase [20,47] and/or topoisomerase [48] inhibition.

There is support in the literature regarding kinase inhibition by genistein [23,47,49–51]. Furthermore, signal transduction pathways involving protein kinases can mediate activation of the AhR, particularly when low-affinity AhR ligands are involved in xenobiotic induction [15,52]. These effects would impair nuclear translocation of AhR and therefore decrease binding to the xenobiotic response element (XRE), which could result in a decrease in cross-talk with ARE transcription factors and a lack of induction in QR gene transcription [15,53]. In our study, genistein (1 and 5 μmol/L) did not increase QR mRNA but did increase Nrf2 binding to the QR ARE, which supports inhibition of AhR complex phosphorylation and translocation and/or topoisomerase inhibition.

Nrf2 is required for both basal and inducible transcription of QR, and induction of QR requires phosphorylation of Nrf2 by upstream kinases and/or stabilization of Nrf2 via cysteine thiol modifications and/or disruption of Nrf2-Keap1 complex by other means, with subsequent translocation of Nrf2 into the nucleus, recruitment of transcription factors and binding to response elements in the gene promoter region [34]. The mechanism by which the AhR participates in QR induction is not well understood. More studies need to be done regarding protein factors mediating the induction of phase II genes via the AhR and potential cross-talk between the XRE and ARE dependent signal transduction pathways. Furthermore, an ARE and XRE have been discovered in the mouse and are close in proximity. This suggests a functional overlap between the enhancers where Nrf2 and AhR bind and mediate induction of QR [1,20,54], interacting directly or through adaptor proteins [1,55–57].

In a recent study [58], Nrf2 mRNA and protein levels were not altered in rats fed 2000 mg/kg genistein for 3 wk. It was suggested that the induction of QR is not mediated via an Nrf2-dependent pathway. However, in the current study, we observed an increase in Nrf2 binding to the QR ARE in response to treatment with genistein, daidzein and equol. Our ChIP results indicate that daidzein and equol modulate QR via both Nrf2 and ERβ strongly binding to the ARE similar to anti-estrogens, whereas genistein stimulates Nrf2 binding and, to a lesser extent, ERβ binding. This later result is consistent with the manner in which catechol estrogens mediate activation of the QR ARE via upstream kinase phosphorylation and stimulation of Nrf2 nuclear translocation without involvement of ER [59].

In conclusion, soy isoflavones increase QR activity, protein, and mRNA at physiologic and supraphysiologic concentrations in Hepa-1c1c7 cells. The molecular mechanisms by which genistein, daidzein and equol interact with the QR antioxidant response element differs, with daidzein and equol modulating QR via both ERβ and Nrf2 binding strongly to the QR ARE, and genistein acting predominantly via Nrf2, with less involvement of ERβ. This study extends previous knowledge by elucidating mechanisms for the induction of QR, thereby describing a process by which isoflavones can decrease the risk for cancer.

References

[1] Ma Q, Kinneer K, Bi Y, Chan JY, Kan YW. Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-p-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction. Biochem J 2004;377(Pt 1):205–13.
[2] Talalay P, Dinkova-Kostova AT. Role of nicotinamide quinone oxidoreductase 1 (NQO1) in protection against toxicity of electrophiles and reactive oxygen intermediates. Methods Enzymol 2004;382:355–64.
[3] Wattenberg LW, Coccia JB, Lam LK. Inhibitory effects of phenolic compounds on benzo(a)pyrene-induced neoplasia. Cancer Res 1980;40(8 Pt 1):2820–3.
[4] Benson AM, Hunkeler MJ, Talalay P. Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. Proc Natl Acad Sci U S A 1980;77(9):5216–20.
[5] Long II DJ, Gaikwad A, Multani A, Pathak S, Montgomery CA, Gonzalez FJ, et al. Disruption of the NAD(P)H:quinone oxidoreductase 1 (NQO1) gene in mice causes myelogenous hyperplasia. Cancer Res 2002;62(11):3030–6.
[6] Larson RA, Wang Y, Banerjee M, Wiemels J, Hartford C, Le Beau MM, et al. Prevalence of the inactivating 609C–>T polymorphism in the NAD(P)H:quinone oxidoreductase (NQO1) gene in patients with primary and therapy-related myeloid leukemia. Blood 1999;94(2):803–7.
[7] Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 2003;43:233–60.

[8] Uda Y, Price KR, Williamson G, Rhodes MJ. Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells in vitro by flavonoids. Cancer Lett 1997;120(2):213–6.
[9] Prochaska HJ, Santamaria AB, Talalay P. Rapid detection of inducers of enzymes that protect against carcinogens. Proc Natl Acad Sci U S A 1992;89(6):2394–8.
[10] Ricketts ML, Moore DD, Banz WJ, Mezei O, Shay NF. Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. J Nutr Biochem 2005;16(6):321–30.
[11] Moon YJ, Wang X, Morris ME. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro 2006;20(2):187–210.
[12] Li Y, Mezei O, Shay NF. Human and murine hepatic sterol-12-alpha-hydroxylase and other xenobiotic metabolism mRNA are upregulated by soy isoflavones. J Nutr 2007;137(7):1705–12.
[13] Singhal R, Badger TM, Ronis MJ. Rats fed soy protein isolate (SPI) have impaired hepatic CYP1A1 induction by polycyclic aromatic hydrocarbons as a result of interference with aryl hydrocarbon receptor signaling. Toxicol Appl Pharmacol 2008;227(2):275–83.
[14] Appelt LC, Reicks MM. Soy feeding induces phase II enzymes in rat tissues. Nutr Cancer 1997;28(3):270–5.
[15] Yannai S, Day AJ, Williamson G, Rhodes MJ. Characterization of flavonoids as monofunctional or bifunctional inducers of quinone reductase in murine hepatoma cell lines. Food Chem Toxicol 1998;36(8):623–30.
[16] Bolling BW, Parkin KL. Phenolic derivatives from soy flour ethanol extract are potent in vitro quinone reductase (QR) inducing agents. J Agric Food Chem 2008; 56(22):10473–80.
[17] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236 (2):313–22.
[18] Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD (P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A 1996;93(25): 14960–5.
[19] Nguyen T, Huang HC, Pickett CB. Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. J Biol Chem 2000; 275(20):15466–73.
[20] Jaiswal AK. Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med 2004;36(10):1199–207.
[21] Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3(10):768–80.
[22] Hernandez-Montes E, Pollard SE, Vauzour D, Jofre-Montseny L, Rota C, Rimbach G, et al. Activation of glutathione peroxidase via Nrf1 mediates genistein's protection against oxidative endothelial cell injury. Biochem Biophys Res Commun 2006;346 (3):851–9.
[23] Gradin K, Whitelaw ML, Toftgard R, Poellinger L, Berghard A. A tyrosine kinase-dependent pathway regulates ligand-dependent activation of the dioxin receptor in human keratinocytes. J Biol Chem 1994;269(38):23800–7.
[24] Bianco NR, Chaplin LJ, Montano MM. Differential induction of quinone reductase by phytoestrogens and protection against oestrogen-induced DNA damage. Biochem J 2005;385(Pt 1):279–87.
[25] Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79(5):727–47.
[26] Erdman Jr JW, Badger TM, Lampe JW, Setchell KD, Messina M. Not all soy products are created equal: caution needed in interpretation of research results. J Nutr 2004;134(5):1229S–33S.
[27] Yellayi S, Zakroczymski MA, Selvaraj V, Valli VE, Ghanta V, Helferich WG, et al. The phytoestrogen genistein suppresses cell-mediated immunity in mice. J Endocrinol 2003;176(2):267–74.
[28] Xu X, Harris KS, Wang HJ, Murphy PA, Hendrich S. Bioavailability of soybean isoflavones depends upon gut microflora in women. J Nutr 1995;125(9): 2307–15.
[29] Klein CB, King AA. Genistein genotoxicity: critical considerations of in vitro exposure dose. Toxicol Appl Pharmacol 2007;224(1):1–11.
[30] Setchell KD, Brown NM, Desai PB, Zimmer-Nechimias L, Wolfe B, Jakate AS, et al. Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. J Nutr 2003;133(4):1027–35.
[31] Prochaska HJ, Santamaria AB. Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. Anal Biochem 1988;169(2):328–36.
[32] Korashy HM, El-Kadi AO. Regulatory mechanisms modulating the expression of cytochrome P450 1A1 gene by heavy metals. Toxicol Sci 2005;88(1):39–51.
[33] Nioi P, McMahon M, Itoh K, Yamamoto M, Hayes JD. 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 2003;374(Pt 2):337–48.
[34] Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. Planta Med 2008;74(13):1526–39.
[35] Mirsalis JC, Hamilton CM, Schindler JE, Green CE, Dabbs JE. Effects of soya bean flakes and liquorice root extract on enzyme induction and toxicity in B6C3F1 mice. Food Chem Toxicol 1993;31(5):343–50.
[36] Appelt LC, Reicks MM. Soy induces phase II enzymes but does not inhibit dimethylbenz[a]anthracene-induced carcinogenesis in female rats. J Nutr 1999; 129(10):1820–6.
[37] Froyen EB, Reeves JR, Mitchell AE, Steinberg FM. Regulation of phase II enzymes by genistein and daidzein in male and female Swiss Webster mice. Journal of Medicinal Food. 2009(in press).
[38] Wang W, Liu LQ, Higuchi CM, Chen H. Induction of NADPH:quinone reductase by dietary phytoestrogens in colonic Colo205 cells. Biochem Pharmacol 1998;56(2): 189–95.
[39] Setchell KD, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. J Nutr 2002;132(12):3577–84.
[40] Peng WX, Li HD, Zhou HH. Effect of daidzein on CYP1A2 activity and pharmacokinetics of theophylline in healthy volunteers. Eur J Clin Pharmacol 2003;59(3):237–41.
[41] Rowland IR, Wiseman H, Sanders TA, Adlercreutz H, Bowey EA. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr Cancer 2000;36(1):27–32.
[42] Morito K, Hirose T, Kinjo J, Hirakawa T, Okawa M, Nohara T, et al. Interaction of phytoestrogens with estrogen receptors alpha and beta. Biol Pharm Bull 2001;24 (4):351–6.
[43] Hwang CS, Kwak HS, Lim HJ, Lee SH, Kang YS, Choe TB, et al. Isoflavone metabolites and their in vitro dual functions: they can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J Steroid Biochem Mol Biol 2006;101(4-5):246–53.
[44] Ansell PJ, Lo SC, Newton LG, Espinosa-Nicholas C, Zhang DD, Liu JH, et al. Repression of cancer protective genes by 17beta-estradiol: ligand-dependent interaction between human Nrf2 and estrogen receptor alpha. Mol Cell Endocrinol 2005;243(1-2):27–34.
[45] Setchell KD. Soy isoflavones — benefits and risks from nature's selective estrogen receptor modulators (SERMs). J Am Coll Nutr 2001(5 Suppl):354S–62S discussion 81S-83S.
[46] Nguyen T, Yang CS, Pickett CB. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. Free Radic Biol Med 2004;37(4):433–41.
[47] Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 1987;262(12): 5592–5.
[48] Kikuchi H, Hossain A, Yoshida H, Kobayashi S. Induction of cytochrome P-450 1A1 by omeprazole in human HepG2 cells is protein tyrosine kinase-dependent and is not inhibited by alpha-naphthoflavone. Arch Biochem Biophys 1998;358(2): 351–8.
[49] Huang X, Chen S, Xu L, Liu Y, Deb DK, Platanias LC, et al. Genistein inhibits p38 map kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells. Cancer Res 2005;65(8):3470–8.
[50] Li Y, Sarkar FH. Gene expression profiles of genistein-treated PC3 prostate cancer cells. J Nutr 2002;132(12):3623–31.
[51] Takahashi Y, Lavigne JA, Hursting SD, Chandramouli GV, Perkins SN, Barrett JC, et al. Using DNA microarray analyses to elucidate the effects of genistein in androgen-responsive prostate cancer cells: identification of novel targets. Mol Carcinog 2004;41(2):108–19.
[52] Backlund M, Ingelman-Sundberg M. Regulation of aryl hydrocarbon receptor signal transduction by protein tyrosine kinases. Cell Signal 2005;17(1):39–48.
[53] Miao W, Hu L, Scrivens PJ, Batist G. Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. J Biol Chem 2005;280(21):20340–8.
[54] Nioi P, Hayes JD. Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. Mutat Res 2004;555(1-2):149–71.
[55] Chan WK, Yao G, Gu YZ, Bradfield CA. Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways. Demonstration of competition and compensation. J Biol Chem 1999;274(17):12115–23.
[56] Ke S, Rabson AB, Germino JF, Gallo MA, Tian Y. Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide. J Biol Chem 2001;276(43):39638–44.
[57] Elferink CJ, Ge NL, Levine A. Maximal aryl hydrocarbon receptor activity depends on an interaction with the retinoblastoma protein. Mol Pharmacol 2001;59(4): 664–73.
[58] Wiegand H, Wagner AE, Boesch-Saadatmandi C, Kruse HP, Kulling S, Rimbach G. Effect of dietary genistein on phase II and antioxidant enzymes in rat liver. Cancer Genomics Proteomics 2009;6(2):85–92.
[59] Lee JM, Anderson PC, Padgitt JK, Hanson JM, Waters CM, Johnson JA. Nrf2, not the estrogen receptor, mediates catechol estrogen-induced activation of the antioxidant responsive element. Biochim Biophys Acta 2003;1629(1-3):92–101.