Published in final edited form as:

Breast Cancer Res Treat. 2010 November ; 124(2): 585–591. doi:10.1007/s10549-010-1023-8.

Inhibition of estrogen signaling activates the NRF2 pathway in breast cancer

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Abstract

Exposure to higher levels of estrogen produces genotoxic metabolites that can stimulate mammary tumorigenesis. Induction of NF-E2-related factor 2 (NRF2)-dependent detoxifying enzymes (e.g., NAD(P)H-quinone oxidoreductase 1 (NQO1)) is considered an important mechanism of protection against estrogen-associated carcinogenesis because they would facilitate removal of toxic estrogens. Here, we studied the impact of estrogen-receptor (ER) signaling on NRF2-dependent gene transcription. In luciferase assay experiments using the 5-flanking region of the human *NQO1* gene promoter, we observe that ER α ligand-binding domain (LBD) is required for estrogen inhibition of *NQO1* promoter activity in estrogen-dependent breast cancer cells. Chromatin immunoprecipitation (ChIP) assay shows that estrogen recruits ER α and a class III histone deacetylase SIRT1 at the *NQO1* promoter, leading to inhibition of *NQO1* transcription. Inhibition of ER α expression by the antiestrogen shikonin reverses the inhibitory effect of estrogen on *NQO1* expression. As a consequence, a chemoprevention study was undertaken to monitor the impact of shikonin on DNA lesions and tumor growth. Treatment of MCF-7 breast cancer cells with shikonin inhibits estrogen-induced 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of DNA damage. *NQO1* deficiency promotes estrogen-dependent tumor formation, and shikonin inhibits estrogen-dependent tumor growth in an NQO1-dependent manner in MCF-7 xenografts. These results suggest that estrogen-receptor signaling pathway has an inhibitory effect on NRF2-dependent enzymes. Moreover, shikonin reverses the inhibitory effects of estrogen on this pathway and may contribute to breast cancer prevention.

Keywords

Estrogen receptor; NRF2; NQO1; Chemoprevention

Introduction

Estrogen is a key carcinogenic factor for the development of breast cancer. One potential mechanism for estrogen carcinogenesis lies in its oxidation to semiquinones and quinones by cytochrome 450 enzymes including CYP1A1, 1A2, 1B1 and 3A [1]. These oxidative metabolites further react with DNA to form stable and unstable DNA adducts leading to mutation [1, 2]. In addition, oxidative estrogen metabolites generate reactive oxygen species (ROS) that can cause DNA damage and permanent mutations [3]. The role of ER α in the genesis of breast cancer has been characterized. There has been considerable effort to reveal that ER α -mediated estrogen signaling contributes to mammary tumorigenesis [1]. ER α also promotes transportation of genotoxic estrogen metabolites into the nucleus and subsequently enhances oxidative DNA damage [4].

Several detoxifying enzymes including NQO1 and glutathione *S*-transferases (GSTs) may protect against estrogen-associated carcinogenesis by removal of genotoxic metabolites of estradiol [1, 3]. The transcription factor NRF2 binds to the antioxidant response element (ARE) in the promoters of genes encoding these detoxification enzymes to increase transcription [5, 6]. However, NRF2 function is inhibited by association with a negative regulator protein, Kelch-like ECH-associated protein 1 (Keap1) that facilitates NRF2 ubiquitination and degradation [6]. An important strategy in cancer chemoprevention is to increase NRF2 levels. This leads to enhanced interaction of NRF2 with ARE in NRF2 dependent gene promoters and activation of genes encoding detoxifying enzymes including NQO1 and GSTs [7].

The important role of estrogen in mammary tumori-genesis has led to the development of antiestrogen therapies through binding of antagonist molecules to ER α and inhibition of estrogen-mediated gene expression. Several studies demonstrated that antiestrogen agents stimulate *NQO1* transcription and protect against estrogen-induced DNA damage in estrogen-dependent breast cancer cells [8, 9]. The selective estrogen receptor modulators (SERM), such as tamoxifen, preferentially act on ER β , which binds to the *NQO1* promoter and subsequently activates *NQO1* gene transcription in breast cancer cells [8, 9]. Although previous studies showed that ER α also binds to an ARE in the *NQO1* promoter [10-12], it is not clear if ER α is equally important in antiestrogen activation of NQO1. In previous studies, the authors have demonstrated that shikonin (a bioactive agent extracted from the Shikon plant) is a potent inhibitor of estrogen-initiated signaling in breast cancer cells [13]. Shikonin does not directly bind to ER α but induces ER α protein degradation, which leads to inhibition of ER α binding at the estrogen-dependent gene promoters and suppression of estrogen-mediated gene expression (c-myc, PR and cyclin D1) [13]. The chemopreventive potential of shikonin in breast cancer has not been investigated. In this study, we examine the impact of shikonin on NQO1 transcription and relevant chemopreventive responses in estrogen-dependent human breast cancer cells.

Materials and methods

Cell culture and reagents

Human breast cancer cells MCF-7, T47D, and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% glutamine (Invitrogen, Carlsbad, CA). Cells were incubated

at 37°C in an atmosphere containing 5% CO₂. Shikonin was purchased from BIOMOL (Plymouth Meeting, PA). 17 β -Estradiol (E2) was obtained from Sigma (St. Louis, MO).

Luciferase reporter assay

Cells were seeded in six-well plates (5×10^5 /well) and co-transfected with human *NQO1*-ARE luciferase plasmids [14] plus full-length or mutant ER α expression vectors [15-17] using Lipofectamine 2000 (Invitrogen). After 48-h posttransfection, cells were treated with or without estrogen for 24 h. Effects of estrogen on *NQO1* promoter activity were determined by a dual luciferase assay (Promega, Madison, WI). Luciferase activity was normalized to *Renilla* luciferase activity. The mean value and SE were calculated. Data were analyzed using One-way ANOVA followed by Bonferroni's *t* test. $P < 0.05$ was considered significant.

Protein isolation and immunoblotting

Cultured cells were lysed in buffer containing 1% SDS and 10 mM Tris-HCl (pH 7.4). Proteins were denatured in SDS sample buffer and separated by 10% SDS-PAGE. Separated proteins were transferred to PVDF membrane. Membranes were incubated with antibodies (NRF2 and NQO1) from Santa Cruz Biotechnology (Santa Cruz, CA) followed by incubation with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz). Immunoreactive proteins were visualized using chemiluminescence (ECL, Amersham, Piscataway, NJ).

mRNA isolation and quantitative real time-PCR

Total mRNA was isolated and cDNA was synthesized using an oligo(dT) primer. Primer sequences of *NQO1* for quantitative real time-PCR were AGGCTGGTTTGAGCGAGTTC and ATTGAATTTCGGCGTCTGCTG. *NQO1* mRNA was measured by SYBR Green PCR Master Mix (Bio-Rad Laboratories, Los Angeles, CA). Quantitative real-time PCR was carried out in Lightcycler 480II (Roche Applied Science, Indianapolis, IN). Relative *NQO1* mRNA was normalized to the GAPDH house-keeping gene and calculated using the $\Delta\Delta C_t$ comparative method as described previously [13, 18].

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed as described in the previous studies by the authors [13, 18]. In brief, cells were cross-linked with 1% formaldehyde, and then sonicated. Soluble chromatin was collected and incubated on a rotating platform with antibodies against ER α , NRF2, and SIRT1 (Santa Cruz) overnight at 4°C. In order to confirm antibody specificity, relevant IgG subtype was used as a negative control. The DNA was recovered and subjected to PCR analysis using primers flanking the ARE of the human *NQO1* gene promoter.

Flow cytometry assay for 8-hydroxy-2'-deoxyguanosine (8-OHdG)

For 8-OHdG labeling, 1×10^6 cells were fixed in $1 \times$ PBS with 4% paraformaldehyde. After washing in washing buffer, cells were stained with fluorescein isothiocyanate (FITC)-conjugated 8-OHdG antibodies (Biotrin OxyDNA test Kit, Biotrin International Ltd, Ireland) as previously described [19]. In parallel, cells were stained with FITC-conjugated anti-IgG antibody (subtype-matched anti-8-OHdG antibody as control). Fluorescently labeled cells were examined by flow cytometry. Data were analyzed using WinMid version 2.8 software (Scripts Institute, La Jolla, CA).

MCF-7 xenografts

In order to establish estrogen dependent human breast cancer tumors, female ovariectomized athymic *nu/nu* mice (4–6 weeks of age, National Cancer Institute, Frederick, MD) were treated with E₂ for the duration of the experiment as previously described [20]. In order to determine the role of *NQO1* in estrogen-dependent tumor growth, Lentivirus was generated by cotransfection of *NQO1* shRNA plasmids with packaging constructs (pCMV-VSV-G and pCMV-dR8.2 dvpr) into HEK293T cells. *NQO1* shRNA plasmids were kindly provided by Dr. Yosef Shaul (Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel). *NQO1* shRNA knockdown was confirmed by real time-PCR and Western blot analysis. The wild-type and *NQO1*-deficient MCF-7 cells were washed twice with cold HBSS and 2×10^7 cells were mixed with 10 mg/ml Matrigel Reduced Factors (BD Biosciences, San Jose, CA) and inoculated into both sides of mammary fat pad with 100 μ l of cell suspension. Mice were housed under controlled temperature, humidity, and lighting conditions. All procedures were approved by the University of Maryland Animal Care and Use Committee. The resulting ER α -positive tumor xenografts have been extensively used to study antihormone therapy and simulate the postmenopausal breast cancer patients who maintain estrogen from nonovarian tissues [21, 22]. On the day after tumor cells were implanted, animals were randomized to receive vehicle DMSO or shikonin (8 mg/kg per day) by oral gavage. Tumors were monitored weekly for 4 weeks. At termination of the study, mice were anesthetized with isoflurane inhalation before cervical dislocation. Tumors were excised, cleaned, weighted and stored in liquid nitrogen for later analysis.

Results

ER α LBD is important for estrogen suppression of *NQO1* promoter activity

The impact of estrogen on *NQO1* promoter activity was examined in the estrogen dependent human breast cancer line MCF-7. As shown in Fig. 1a, treatment with estrogen inhibited *NQO1* promoter luciferase activity in a concentration-dependent manner, suggesting that estrogen-bound ER α may negatively regulate *NQO1* transcription. In order to gain an insight into the mechanism by which ER α contributes to estrogen suppression of *NQO1* transcription, ER α negative MDA-MB-231 cells were co-transfected with *NQO1* promoter luciferase construct with full-length or mutant ER α cDNAs. Figure 1b shows the effects of the variant ER α constructs including the complete removal of LBD, the hinge region or the DNA binding domain (DBD) on reporter activities. Estrogen-mediated reduction of *NQO1* promoter activity was observed in cells transfected with full-length ER α cDNA. E2-dependent suppression of *NQO1* promoter activity was partially reversed when the complete LBD was deleted. Deletion of the hinge region, DBD and AF1 domain had modest impact on the effects of estrogen action, suggesting that the presence of ER α LBD is critical for estrogen-receptor binding, which in turn enables estrogen to inhibit *NQO1* promoter activity.

Inhibition of estrogen–receptor-signaling enhances *NQO1* promoter activity

Our previous studies showed that shikonin inhibits estrogen response by depletion of ER α expression in breast cancer cells [13]. In order to test the hypothesis that ER α may act as a repressor to NRF2-dependent *NQO1* transcription, MCF-7 cells were transfected with either control or *NQO1*-ARE promoter luciferase plasmids followed by treatment with shikonin for 24 h. *NQO1* promoter luciferase activity was increased by treatment with shikonin (1 and 2 μ M) (Fig. 2a). The effect of shikonin on ER α expression was examined by Western blot analysis. As shown in Fig. 2b, treatment with shikonin completely depleted ER α protein by 24 h. This effect is associated with elevated levels of NRF2 protein and induction of NQO1 protein. Keap1 protein (a cytoplasmic repressor of NRF2) levels were not changed

in response to shikonin treatment. These data suggest that reduction of ER α by shikonin contributes to activation of NRF2-dependent *NQO1* transcription.

Shikonin inhibits ER α and SIRT1 association with the *NQO1* promoter and reverses estrogen suppression of *NQO1* mRNA

The *NQO1* promoter contains a specific ER α binding region within the ARE that contributes to antiestrogen activation of *NQO1* [12]. ChIP was used to monitor binding of transcriptional factors to the endogenous *NQO1* promoter. Following immunoprecipitation using anti-ER α , NRF2 or SIRT1 antibodies, DNA was recovered and subjected to PCR analysis using oligonucleotide primers flanking the ARE of the human *NQO1* gene promoter. Figure 2c shows that estrogen recruits ER α and SIRT1 (a class III histone deacetylase) to the *NQO1* promoter. RT-PCR analysis shows that estrogen abrogates *NQO1* mRNA expression, whilst treatment with shikonin reversed E2 inhibition by enhancing NRF2 binding and disrupting the association of ER α and SIRT1 with the promoter (Fig. 2d). This effect is associated with enhanced induction of *NQO1* mRNA (Fig. 2d). These results indicate that shikonin activates *NQO1* transcription by altering the association of proteins at the *NQO1* promoter. In order to further characterize the role of shikonin on the activation of *NQO1* transcription in estrogen dependent human breast cancer cells, the effects of shikonin on *NQO1* mRNA were monitored by quantitative real-time PCR analysis. As shown in Fig. 3, treatment with shikonin (1 and 2 μ M) increased *NQO1* mRNA in MCF-7 cells at 24 h. Similar results were observed in T47D cells. These data suggest that shikonin enhances *NQO1* promoter activity, leading to induction of *NQO1* mRNA expression.

Shikonin inhibits estrogen-induced DNA damage

Activation of *NQO1* can increase stability of the tumor suppressor p53 through an ubiquitin-independent mechanism [23, 24]. We therefore examined if p53 protein levels increase with *NQO1* activity. The results show that shikonin increased p53 accumulation in a time-dependent manner, with a marked effect at 48 h (Fig. 4a). This suggests that induction of *NQO1* and p53 by shikonin may contribute to protection. Formation of 8-OHdG is an indicator of oxidative DNA damage in E2-treated breast cancer cells [25]. We used flow cytometry analysis to detect 8-OHdG as a marker of oxidative DNA damage in MCF-7 cells (Fig. 4b). Background IgG staining was used as a negative control. No differences in 8-OHdG staining between vehicle control- and shikonin-treated cells were observed, suggesting that treatment with shikonin alone did not induce oxidative DNA damage. However, treatment with 20 nM E2 resulted in increased levels of 8-OHdG. Figure 4b shows that shikonin dramatically reduces E2-induced accumulation of 8-OHdG. These results suggest that shikonin could inhibit the genotoxicity of estrogen mediated by formation of reactive oxygen species.

Shikonin prevents tumor growth in MCF-7 xenografts

In order to translate our in vitro findings into an in vivo model, we examined the effect of shikonin on estrogen dependent tumor growth model using two forms of MCF-7 xenografts: wild-type and *NQO1*-deficient. Treatments began the day after tumor cell implantation, and animals were randomized to receive vehicle (DMSO) or shikonin (8 mg/kg per day) by oral gavage, a regimen that has been shown previously to inhibit growth of H22 (murine hepatoma cells) and PC-3 (prostate cancer cells) xenografts [26]. As shown in Fig. 5, tumors from *NQO1*-deficient MCF-7 xenografts grew significantly better than wild-type MCF-7 xenografts. The weight of tumors from wild-type but not *NQO1*-deficient MCF-7 cells was significantly inhibited after 4 weeks of treatment with shikonin. These data suggest that (a) the presence of *NQO1* protects against estrogen dependent tumor formation and (b) that shikonin inhibits estrogen dependent tumor growth in an *NQO1* dependent manner.

Discussion

NQO1 has been shown to protect against the toxicity and carcinogenicity of estrogen metabolites [8, 25]. Recent studies suggest that *NQO1* deficiency is associated with cancer progression and chemotherapy resistance; moreover, NQO1 levels have been suggested to be important prognostic and predictive markers in breast cancer [27]. Thus, activation of NRF2-dependent NQO1 transcription may play a critical role in chemoprevention and chemotherapy of breast cancer.

In agreement with previous studies, the authors demonstrated that exposure of MCF-7 cells to estrogen suppresses NQO1 expression and induces DNA damage [25]. We found that estrogen acts on ER α ligand binding domain, and inhibits NQO1 promoter activity. We observed that ER α and SIRT1 (a member of class III HDACs) are associated with the *NQO1* promoter in response to estrogen stimulation. These results provide evidence that ER α and SIRT1 may act as repressors leading to inactivation of *NQO1* transcription. Further study is needed to understand how ER α and SIRT1 regulate *NQO1* promoter activity with regard to cancer cell type, tissue, and chromatin-associated proteins (e.g., histone modifications).

The data of this study show that higher levels of estrogen reduce expression of the key detoxifying enzyme, NQO1, and subsequently generate oxidative DNA damage in ER α -positive breast cancer cells, supporting the hypothesis that ER α may facilitate estrogen genotoxicity [4, 28]. Estrogen has been shown to induce DNA damage and to stimulate mammary tumorigenesis in the August Copenhagen Irish (ACI) rats [8]. Results from this study and other laboratories support the view that the estrogen–receptor–signaling pathway plays an important role in the suppressed expression of detoxifying enzymes, and that inhibition of estrogen–receptor signaling may prevent breast cancer development by induction of cytoprotective enzymes.

The important role of estrogen in breast carcinogenesis has led to the development of chemoprevention strategies that inhibit ER α signaling and enhance detoxification enzyme-dependent removal of genotoxic estrogen metabolites. Conventional anti-estrogen agents that block the estrogen action are used to prevent disease in women at risk for breast cancer, but they may be associated with development of drug resistance and increased risk of endometrial cancer [29, 30]. This suggests that these agents may not provide optimal prevention for this disease. For this reason, we have initiated studies on the dietary agent, shikonin [31, 32], with the idea that lifelong consumption of this agent may reduce cancer risk without producing negative side effects. Based on our previous studies, we hypothesize that shikonin may exert its chemopreventive properties through activation of detoxifying enzymes. Our results show that shikonin inhibits estrogen–receptor–signaling, which subsequently leads to activation of NRF2-dependent NQO1 transcription. Treatment with shikonin inhibits the association of the transcriptional repressors ER α and SIRT1 at the NQO1 promoter. Thus, we suggest that shikonin activates NQO1 transcription by at least three mechanisms: depletion of ER α , increased NRF2 levels, and formation of a transcriptional complex at the NQO1 promoter. Importantly, the findings in MCF-7 cells in vitro were confirmed in a xenograft study where shikonin inhibits estrogen-dependent MCF-7 xenograft growth.

In conclusion, this study shows that shikonin induces up-regulation of NRF2-dependent NQO1 transcription and attenuates estrogen genotoxicity in estrogen-dependent human breast cancer cells. These data enhance our understanding of the role of ER α in the regulation of detoxifying enzymes and suggest that the inhibition of ER α signaling and

activation of NQO1 may represent a good strategy for prevention of estrogen dependent breast cancer.

Acknowledgments

This study was supported by the Flight Attendants Medical Research Institute (FAMRI YCSA072084 to QZ) and NIH P50 CA088843 (NED and TWK).

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Fig. 1.

a Estrogen treatment inhibits *NQO1* promoter activity. MCF-7 cells (5×10^5 /well in 6-well plates) were co-transfected with the *NQO1*-ARE promoter luciferase plasmids and Renilla luciferase constructs. After 24 h of transfection, cells were grown in DMEM supplemented with 5% charcoal/dextran-stripped FBS for 24 h followed by treatment with E2 (10–20 nM for an additional 24 h). *NQO1* luciferase activity was normalized to Renilla and is shown as the mean $\pm$ SE of three independent experiments. * $P < 0.05$. **b** ER α LBD is important for estrogen inhibition of *NQO1* promoter activity. MDA-MB-231 cells were co-transfected with the *NQO1* promoter luciferase plasmids and full-length or mutant ER α cDNAs. Cells were grown in 20 nM E2 in DMEM supplemented with 5% charcoal/dextran-stripped FBS for 24 h, and the *NQO1* promoter luciferase activity was measured as described above. Values represent mean $\pm$ SE of three independent experiments

Fig. 2.

a Shikonin enhances *NQO1* promoter activity. MCF-7 cells (5×10^5 /well in 6-well plates) were co-transfected with the *NQO1*-ARE promoter luciferase plasmids and Renilla luciferase constructs. After 24 h of transfection, cells were grown in DMEM supplemented with 5% FBS for 24 h and treated with vehicle or 1 or 2 μ M shikonin (SK) for an additional 24 h. **b** Shikonin increases NQO1 expression. MCF-7 cells were treated with shikonin for 24 h and whole cell lysates were immunoblotted with antibodies as indicated. β -actin was used as a loading control. The blots are representative of three independent experiments that all gave similar results. **c** and **d** Shikonin reverses estrogen inhibition of NQO1 transcription. T47D cells were grown in DMEM supplemented with 5% charcoal/dextran-stripped FBS for 24 h prior to start of treatment with 20 nM E2 alone or combination with 1 μ M shikonin for 24 h. ChIP assay was performed as described in "Materials and methods". The purified DNA was analyzed by PCR using specific primers spanning the *NQO1* gene promoter. A representative ChIP from three independent experiments is shown (**c**). *NQO1* mRNA expression was measured by RT-PCR (**d**).

Fig. 3. Shikonin increases levels of *NQO1* mRNA in MCF-7 and T47D cells. Cells were treated with shikonin for 24 h and *NQO1* mRNA was measured by quantitative real time PCR analysis. Values represent the mean $\pm$ SE of four independent experiments

Fig. 4.

a Shikonin (1 μ M SK) increases levels of p53 protein by 48 h in MCF-7 breast cancer cells.

b Shikonin inhibits estrogen-induced DNA damage. MCF-7 cells were treated with 20 nM E2, 1 μ M SK or the combination for 48 h and the DNA damage marker 8-OHdG was measured by flow cytometry. Background IgG staining was used as a negative control

Fig. 5. Shikonin inhibits estrogen-dependent tumor growth through an NQO1-dependent pathway. Wild-type and *NQO1* shRNA knockout MCF-7 cells were xenografted into ovariectomized female athymic nude mice (6 weeks of age) as described in “Materials and methods”. On the day after, tumor cell injection animals began daily treatment with shikonin for 4 weeks. At the end of the study, tumor tissues were collected and weighted ($n = 5$, $P < 0.05$)