1. ABSTRACT

Antiestrogens have found widespread use in the treatment of breast cancer (reviewed in ref. 1). There is also interest in the use of tamoxifen as a preventive agent for breast cancer. However, the mechanism for the antitumor effects of antiestrogens is relatively unknown. For the most part it is thought that the basis for the anticancer action of antiestrogens is the inhibition of estradiol (E2)-stimulated tumor growth. We have observed however that antiestrogens can activate detoxifying enzymes, like quinone reductase (NQO1), which protect cells against the toxic and tumor-promoting effects of carcinogens (2). Studies characterizing the molecular mechanisms behind the regulation of NQO1 by the Estrogen Receptor (ER) support an important role of the ER in pathways regulating antioxidant defenses. Moreover these findings represent a novel mechanism through which antiestrogens function. The activation of NQO1 may contribute to the beneficial anticancer and antioxidant activity of antiestrogens in breast cancer and possibly other estrogen target tissues. It is possible that the basis for some of the anticancer action of antiestrogens is that the induction of NQO1 inhibits the genotoxic effects induced by the oxidative metabolism of estrogens.

2. INTRODUCTION

2.1. Estrogen receptor bioactivity in breast cancer cells
The estrogen receptor (ER) protein is essential for mediating the actions of estrogen in target tissues. The binding of estrogen initiates a process of receptor activation that includes the high affinity binding of ER to specific DNA sequences, termed estrogen response elements (EREs). The interaction of ER with EREs results in the modulation of specific gene expression, through which the physiological actions of estrogens are manifested (figure 1A; reviewed in ref. 3). Estrogens acting via the ER dramatically escalate proliferative and metastatic activity in these tumor cells, in part via the induction of growth factors, proteases, and basement membrane receptors (reviewed in ref. 4, 5). However the relative role of the induction of these genes on the proliferative effects of estrogens remains unknown.

2.2. Estrogen receptor alpha and beta
The human ER-alpha is a 67 kDa protein comprising...
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Figure 1. (A) Mechanism of Estrogen Receptor (ER) action, (B) Functional domains of the ER, (C) Estrogen Receptor ER-alpha and ER-beta.

of 595 amino acids. Deletional and mutational analyses have led to the identification of conserved domains involved in DNA binding, ligand binding, dimerization, protein binding, and transcriptional activation (reviewed in ref. 3). The A/B domain contains transactivation function 1 (figure 1B). A highly conserved DNA binding domain comprises domain C. The hinge domain appears to be important in receptor dimerization, and along with carboxy terminal region of domain C is thought to contain the nuclear localization signal. The E domain is functionally complex and is involved in ligand binding, dimerization, ligand-dependent transactivation, and interaction with coactivators and corepressors. A role in estrogen and antiestrogen discrimination has been proposed for the F domain (6).

Another subtype of the ER, ER-beta has been cloned (figure 1C, ref. 7-9). Human ER-beta shares regions of homology such as the DNA binding domain, while the N-terminal A/B domain, hinge region, the ligand binding domain, and the F domain are distinct between ER-alpha and ER-beta. ER-beta exhibits lower transcriptional response to E2 and TOT when compared to ER-alpha in the context of several estrogen response element (ERE)-containing gene reporter constructs (7-9). However, ER-beta has been reported to show increased TOT agonism from reporter constructs containing an AP1 site (9).

ER-alpha is predominantly expressed in the breast, uterus, and vagina (reviewed in ref.10). Reports indicate a positive correlation between ER-alpha and ER-beta in human breast cancers (reviewed in ref. 11 and 12). Studies correlating ER-beta protein expression with breast tumor grade predict good prognosis for ER-beta positive tumors. ER-beta status also appears to be a good predictor of response to tamoxifen (13-16). However, the significance of ER-beta in breast cancer development and treatment of breast cancer is not clear.

2.3. Antiestrogens and the regulation of estrogen receptor bioactivity in breast cancer cells

Tamoxifen is presumed to exert its antitumor and chemoprotective effects by competitively antagonizing the binding of estradiol (E2) to ER, which ultimately leads to the inhibition of gene transcription and protein synthesis. There is also interest in antiestrogen effects exerted through mechanisms that may not involve inhibition of E2-stimulated activities. For example, tamoxifen is also being assessed as a preventive agent for breast cancer and for other potential benefits, such as protection against cardiovascular disease and osteoporosis (17, 18). It has been proposed that tamoxifen may function as an anticancer drug by acting as an antioxidant (19-21). However, the basis for the antioxidant capabilities of tamoxifen has not been well characterized.

Despite its clear efficacy in the short-term treatment of breast cancer, clinically tamoxifen resistance remains a problem (1). Although ca. 75% of ER-positive tumors respond well to tamoxifen therapy, almost all breast tumors eventually become refractory to the beneficial growth suppressive effects of tamoxifen. Another cause for concern is that although tamoxifen acts primarily as a chemopreventive agent in mammary carcinogenesis, it can induce uterine and liver cell proliferation (22). Thus the development of adjuvant therapy for women undergoing tamoxifen therapy is of clinical importance.

2.4. Detoxifying enzymes and chemoprotection against cancer

Detoxifying enzymes such as NAD(P)H:(quinone-acceptor) oxidoreductase [quinone reductase (NQO1)], glutathione S-transferases (GSTs), epoxide hydrolase, and UDP-glucuronosyltransferases are induced in cells by electrophilic compounds and phenolic antioxidants (reviewed in ref. 23 and 24). These widely distributed enzymes detoxify electrophilic quinones, thereby protecting cells against the toxic and neoplastic effects of carcinogens (figure 2). The detoxification mechanism for quinones involves two electron reduction, which bypasses semiquinone generation and thereby diminishes the subsequent production of reactive oxygen species.
In addition to detoxification of exogenous quinones, NQO1 may also play a role as an antioxidant by reducing endogenous quinones and protect cellular membranes against oxidative DNA damage (reviewed in ref. 25). NQO1 can catalyze the reduction of ubiquinone analogs (coenzyme Q) to their ubiquinol forms. The product of Vitamin E (α-tocopherol) oxidation, α-tocopherolquinone, is reduced to α-tocopherolhydroquinone, in a reaction catalyzed by NQO1. This process is able to protect cells against lipid peroxidation.

Depending on the properties of the hydroquinone generated after reduction by NQO1, two electron reduction of a quinone may also represent a bioactivation step. Hydroquinones may autooxidize, leading to the generation of reactive oxygen species and toxicity. Alternatively hydroquinones may undergo rearrangement to produce reactive alkylating agents. This process is termed bioreductive alkylation (26, 27) and bioreductive alkylating agents are of clinical interest for their anticancer effects or their ability to induce DNA damage and toxicity in tumor cells. Thus, NQO1 has attracted a lot of attention due to its ability to metabolize chemothe rapeutic quinones and other bioreductive antitumor compounds in many cells including mammary tumor cells (28-30). Moreover, the levels of detoxifying enzymes like NQO1 and GST have been shown to be elevated in a number of tumor types including breast, liver, and prostate when compared to normal cells of the same origin (31-33). These observations may provide a basis for targeting these tumor types for treatment with chemotherapeutic quinones.

The non-protein thiol, glutathione (gamma-glutamyl-cysteinyl-glycine, GSH) is a predominant cellular antioxidant. As such GSH serves critical functions in the maintenance of cellular redox balance, provides protection against reactive oxygen species (ROS), and is involved in the detoxification of xenobiotics. GSH detoxifies either through direct reactions with reactive intermediates or via enzymatic conjugation reactions catalyzed by GSTs (34). Exposure of cells to a number of xenobiotic agents results in a significant increase in the total intracellular GSH content, secondary to transcriptional up-regulation of the genes encoding the two protein subunits (catalytic and regulatory) of gamma-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in its de novo synthesis (34).

GSTs are important in the detoxification of xenobiotics, catalyzing the nucleophilic attack by the thiol group of GSH on the xenobiotic (35). Since they catalyze the inactivation of several known carcinogens, these enzymes can provide a defense against carcinogenesis. On the other hand, elevation of GST levels in solid tumors appears to be a major factor in the development of resistance to treatment with cytotoxic agents (36). GSTs are

![Diagram of oxidative stress pathways and antioxidant defenses](image-url)
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2.5. Regulation of the expression of detoxifying enzymes

Determination of the molecular mechanisms involved in the induction of detoxifying enzymes is important in devising strategies for chemoprotection against cancer. It has been reported that the induction of genes NQO1, GCSH, and GST is mediated through an electrophile (or antioxidant) response element (EpRE or ARE) (37-40). An EpRE/ARE has been identified in the 5'-flanking region of the human and rat NQO1 genes and the rat GST gene (41, 42). A short core sequence, 5' TGACnnnGC-3', which is present in the EpRE of both NQO1 and GST genes, is required to control gene expression in response to phenolic antioxidants (tert-butylnhydroquinone and hydroquinone), and metabolizable planar aromatic compounds leading to enzyme induction by these compounds (38, 42).

The human NQO1 gene EpRE core sequence (TGACTCAGC) contains a TPA response element or TRE (TGACTCA, ref. 43). The TRE is the binding site for the AP1 family of transcription factors, which includes c-jun and c-fos. However the EpRE sequence of the rat NQO1 gene does not contain a high affinity recognition site for in vitro translated c-jun and c-fos (44). Furthermore, the EpRE of the rat GST Ya gene forms a complex with protein factors in HepG2 cell nuclear extracts that is not competed with the TRE (45). This finding suggests that there are factors in addition to the AP-1 proteins that regulate expression via EpRE sequences. It has been reported that the transcription factors, Nrf1 and Nrf2, are part of the complex that binds to the EpRE and overexpression of these factors in certain cell lines increased EpRE-mediated expression of the NQO1 gene (46).

The transcriptional up-regulation of the two GCS subunit genes involves the EpRE but distinct combinations of trans-acting factors, contributing to differential regulation in response to specific inducing agents (47, 48). Transcription is hypothesized to involve dimeric transcription factors composed of small Maf proteins and various other bZIP family members, including Nrf1 and Nrf2 (47, 48). The AP1 transcription factors, Fos and Jun, have been implicated in the transcriptional regulation of the GST-Pi (49).

2.6. Metabolism of estrogens

In both normal and breast cancer cells estrogens may be oxidized by extrahepatic cytochrome P450 enzymes (mainly CYP1A1 and CYP1B1) to hydroxycatecholestrogens and further oxidized to the semiquinone and quinone form (figure 3; ref. 50-52). This is potentially harmful given that the quinone can bind to DNA forming DNA adducts; and redox cycling, involving quinone and...
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Figure 4. Antiestrogens, but not estrogen, induce NAD(P)H:Quinone Oxidoreductase enzyme activity in MCF-7 breast cancer cells. Effect of different compounds on NQO1 activity in MCF-7 cells. Cells were treated with different concentrations of estradiol (E2), trans-hydroxytamoxifen (TOT), tamoxifen (TAM), ICI182,780 (ICI) or tert-butylhydroquinone (TBHQ), or 12-O-tetradecanoylphorbol-13-acetate (TPA). Cells were harvested after 4 days of treatment. Cytosolic extracts were assayed for NQO1 activity as described in "Materials and Methods." Values are the means ± S.E. from three separate experiments. Reprinted with permission from the National Academy of Sciences, U.S.A. (2).

2.7. Role of estrogen metabolites in carcinogenesis

Recent studies indicate that estrogen itself may be a genotoxic mutagen capable of causing chromosomal mutations in animals and cell culture (53, 59, 60), leading to tumorigenesis in various animal models (53, 61). Estrogens also cause MCF10 breast epithelial cells, that lack both ER-alpha and ER-beta, to take on a transformed phenotype (59). This neoplastic transformation is accompanied by genomic alterations, as exhibited by Loss of Heterozygosity and Microsatellite Instability at specific loci in chromosomes 11, 13 and 17. These loci have been reported to be affected in several types of breast lesions. Further support for a role of estrogen metabolites in carcinogenesis is the observation that ERs in human mammary epithelium are localized in cells distinct from those expressing proliferation markers (62). There are synthetic estrogens that strongly activate ER but have decreased catechol estrogen formation and are poorly carcinogenic (53). Thus, initiation may be due not only to ER-mediated proliferation, but also DNA damage due to a combination of estrogen metabolism and preexisting lesions. Once initiated, the ER may then confer a selective advantage to these premalignant cells.

Increases in estrogen metabolism relative to conjugation were observed in breast carcinomas (63). In line with the protective role of antioxidative stress enzymes, polymorphisms in the NQO1 and GST-Pi loci have been associated with increased risk for breast cancer and other types of cancer (64-71). Epigenetic silencing through promoter hypermethylation of GST-pi gene is associated with breast, prostate and renal cancer (72-73).

3. REGULATION OF NQO1 BY THE ESTROGEN RECEPTOR

3.1. Induction of NQO1 activity in breast cancer cells by antiestrogens

Through the technique of differential RNA display, we observed NQO1 to be a species that was present at a much higher level in an MCF7 breast cancer cell subline that had been grown long-term (over 6 months) in the presence of high (10^{-6} M) levels of the antiestrogen trans-hydroxytamoxifen (TOT), as compared to the parental MCF7 cells grown in the absence of TOT (2). Differential expression was verified using Northern blot analyses which indicate that NQO1 RNA was present at 8-times greater abundance in the long-term TOT-maintained cells than in the parental MCF7 cells. A similar increase in NQO1 mRNA expression can be induced in parental MCF7 cells transiently exposed to the antiestrogens TOT and ICI 182,780 (74). No increase in NQO1 mRNA levels was evident in cells treated with E2; E2 even appeared to slightly reduce the control level of NQO1 mRNA in the cells. We therefore examined the ability of antiestrogen to evoke increases in NQO1 enzymatic activity when parental MCF7 cells were treated with antiestrogens.

As shown in figure 4 a 3- to 4-fold increase in NQO1 activity was observed upon treatment with the antiestrogens TOT, tamoxifen, and ICI182,780. The induction of NQO1 activity by antiestrogens occurred in a dose-dependent manner, and maximal stimulation was obtained with a relatively low concentration of the antiestrogens (10^{-8} M for TOT and ICI, ca. 10^{-7} M for TAM). The concentrations of antiestrogen required for stimulation of NQO1 activity were substantially lower than those needed for stimulation by a previously identified potent inducer of NQO1 activity in other systems, tert-butylhydroquinone (TBHQ). Of note, no increase in NQO1 activity was observed in the presence of E2 or the tumor promoter phorbol ester TPA. We have also observed that the antiestrogen-induced increase in NQO1 activity was fully inhibited by E2 (2). These observations suggest that
the modulation of NQO1 activity by antiestrogens is mediated by the ER. In contrast, E₂ did not affect the induction of NQO1 by TBHQ.

3.2. Induction of EpRE enhancer activity by antiestrogens in breast cancer cells

Studies toward understanding the molecular mechanisms for the induction of detoxifying enzymes by antiestrogens were also conducted in view of their importance in devising strategies for chemoprotection against cancer. To examine the possible transcriptional regulation of the NQO1 gene by antiestrogen, a reporter construct which contains the 863 bp 5'-flanking and promoter regions of the human NQO1 gene upstream of the chloramphenicol acetyltransferase (CAT) gene (figure 5A) was introduced into an ER-negative breast cancer cell line, MDA-MB-231 cells (74). In cells cotransfected with an expression vector for the wild type ER-alpha TOT induced an increase in pNQO1-CAT 0.863 reporter activity. E₂ inhibited the TOT-mediated, not TBHQ-mediated, increase in NQO1 transcriptional activity. No increase in CAT activity was evident in cells after treatment with TPA, a known inducer of AP1 activity (75). Cells that were cotransfected with the control expression vector lacking the ER cDNA (pCMV5), with an expression vector for a mutant ER-alpha that has impaired activation function-2 (AF-2) activity (SS545s), or with an expression vector for a mutant ER-alpha that has impaired DNA-binding ability (DBDmut) did not show activation from pNQO1-CAT 0.863 in response to antiestrogens, although the effect of TBHQ was still observed. Thus antiestrogen-mediated activation of NQO1 gene promoter activity requires a transcriptionally active ER-alpha. Treatment with either TOT or TBHQ did not induce transcription from a deletion mutant of the pNQO1-CAT 0.863 reporter construct, denoted pNQO1-CAT-0.365, that lacks the -366 to -863 bp portion of the 5' flanking region. As expected (44), this truncated construct has reduced basal promoter activity. Our results indicate that the region between -0.863 and -0.365 kb of the NQO1 gene is essential for induction by both TOT and TBHQ.

Sequence analysis of the 5'-flanking sequence of the human NQO1 gene indicates the presence of a single copy of the EpRE motif at position -467 to -437 (40, 43). The EpRE motif contains a TRE and a TRE-like element. The EpRE-containing region was introduced upstream of a heterologous promoter, thymidine kinase, and the CAT gene (40). Although this EpRE-containing construct showed significant basal CAT activity in 231 cells, TOT and TBHQ were able to induce transcription 2.2-fold and 3.4-fold, respectively, over basal levels (left portion of figure 5B). Mutation of the perfect TRE element (middle portion of figure 5B) reduced basal CAT activity and eliminated induction by TBHQ and TOT. Mutation of the TRE-like element (right portion of figure 5B) also reduced basal activity and eliminated the TBHQ- and TOT-induced increase in NQO1 gene transcriptional activity. TPA, which increases AP1 activity, did not induce an increase in EpRE-mediated CAT gene expression of the wild type (unmutated) gene construct in these cells. This observation does not appear to be due to the absence of AP1 activity because we have previously shown that TPA induced TRE-mediated CAT reporter gene activity in 231 cells (2).

In contrast to the observations made with antiestrogens, TBHQ-mediated induction of NQO1 gene transcriptional activity did not require the ER and occurred equally well in the presence of functionally inactive ER or in the absence of ER altogether. However the DNA elements required for antiestrogen-mediated induction of NQO1 gene transcriptional activity that we identified through deletion and mutational studies mapped identically with the elements required for TBHQ-mediated induction. Our experiments indicate that NQO1 can be activated by ER-independent and ER-dependent pathways. Activation by antiestrogens depends on the presence of a functional ER and requires much lower doses of compound (10⁻¹⁰ – 10⁻⁸ M) than stimulation by known activators of NQO1 such as TBHQ (10⁻⁶ – 10⁻³ M). While the signals between these two pathways may be integrated at some level, the ER-dependent pathway – which may involve induction or regulation of some protein that regulates the EpRE – must be different in part from the electrophile-dependent pathway utilized by TBHQ, where the ER is not at all required for NQO1 stimulation.

3.3. Regulation of NQO1 gene transcriptional activity by ER-alpha and ER-beta

Although ER-beta exhibits lower transcriptional response to E₂ and TOT when compared to ER-alpha in the context of several estrogen response element (ERE)-containing gene reporter constructs (7-9), ER-beta has been reported to show increased TOT agonism from reporter constructs containing an AP1 site (9). We thus examined ligand activation of ER-alpha and ER-beta from an EpRE, as compared to activation from an ERE (74).

With a reporter construct containing EREs upstream of the promoter for the estrogen-regulated pS2 gene (figure 6), ER-alpha and ER-beta both showed stimulation by E₂ and as expected, ER-beta showed a lower transcriptional response. Interestingly, ER-alpha was weakly stimulated by the antiestrogen TOT and by another antiestrogen, LY 117018 (LY) at this ERE-containing gene construct, whereas the antiestrogens failed to elicit ER-beta transcriptional activity at this ERE-containing gene. The transcriptional response of ER-alpha and ER-beta to these ligands was very different in the context of the NQO1 gene promoter construct pNQO1-CAT 0.863. Of note, although antiestrogens stimulated NQO1 activity via both ER-alpha and ER-beta, the magnitude of increase in NQO1 gene transcriptional activity in response to TOT or LY was higher with ER-beta than with ER-alpha and no stimulation by E₂ was observed via ER-alpha or ER-beta at an EpRE.

It is clear that ligand activation of ER transcriptional activity is highly dependent on the nature of the response element. Antiestrogen ligands which induce very little activity from ER-beta at an ERE, can induce significant activity from ER-beta at an EpRE and, as previously reported, from a TRE (9). It has been proposed that ER-beta regulates transcription from an AP1 site by a direct interaction with Fos and Jun. However our studies suggest that transcriptional activation of the NQO1 gene by TOT is not mediated through Fos and Jun.
Figure 5. Antiestrogen induction of pNQO1-CAT reporter gene activity in estrogen receptor negative cells is mediated by the estrogen receptor and the EpRE. (A) MDA-MB-231 breast cancer cells were transfected with the pNQO1CAT 0.863 or pNQO1CAT 0.365 reporter construct along with an expression vector for the wild type human estrogen receptor (WT-ER-alpha). 231 cells were also transfected with an expression vector for a DNA binding mutant of ER-alpha (ERDBDmut, missing amino acids 185-251), an expression vector for a mutant ER-alpha that lacks activation function-2 activity (ERS554fs), or the empty expression vector missing the estrogen receptor cDNA (pCMV). (B) 231 cells were transfected with an expression vector for ER-alpha along with pNQO1hARE-tk-CAT (containing the region between -476 and -446 of the NQO1 gene promoter introduced upstream of the thymidine kinase promoter in the pBLCAT2 vector), pNQO1hARE1(mut)-tk-CAT (containing a mutated TRE element), or pNQO1hARE1(mut2)-tk-CAT (containing a mutated TRE-like element). The cells were also transfected with the beta-galactosidase internal reporter to correct for transfection efficiency. The cells were then treated with control ethanol vehicle (c) or varying concentrations of TOT, E2, TPA, or TBHQ as indicated for 24 h. Cell extracts were prepared and analyzed for CAT activity and beta-galactosidase activity as described in Experimental Procedures. Values are the means ± S.E. from three separate experiments. Reprinted by permission from The American Society for Biochemistry and Molecular Biology, Inc. (74).
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Figure 6. Induction of ER-alpha activity versus ER-beta activity from an ERE or an EpRE by different ligands. HEC-1 cells were transfected with the (A) (ERE)2-pS2-CAT or (B) pNQO1CAT 0.863 reporter gene constructs. The cells were also transfected with an expression vector for the human estrogen receptor-alpha or estrogen receptor-beta and with a beta-galactosidase internal control reporter to correct for transfection efficiency. Cells were then treated for 24 h with control ethanol vehicle (c), TOT (10^{-7} M), E2 (10^{-8} M), or LY117018 (LY, 10^{-7} M) as indicated. Cell extracts were prepared and analyzed for CAT activity and beta-galactosidase activity as described in Experimental Procedures. Values are the means ± S.E. from three separate experiments. Reprinted by permission from The American Society for Biochemistry and Molecular Biology, Inc. (74).

3.4. Identification of a novel protein factor, hPMC2, involved in the regulation of NQO1 activity by the estrogen receptor

Several aspects of antiestrogen regulation of NQO1 transcriptional activity cannot be attributed solely to ER binding to the EpRE and remain to be investigated. This is especially true in light of our previous observations that (1) the time course of induction of NQO1 enzyme activity is relatively slow (with increases in NQO1 mRNA first detectable at 12-16 h after antiestrogen treatment of MCF7 cells, ref. 2), (2) antiestrogen activation of GST Ya gene transcriptional activity is mediated through an EpRE which is not homologous to the ERE (ref. 2), and (3) the interaction of the ER with the EpRE is weak and that the EpRE interacts with additional proteins (ref. 74). Clearly the regulation by antiestrogen-ligated ER may be also attributable to changes in the levels and/or the activity of other factors. We used yeast genetic screenings to identify a protein factor, hPMC2 that fulfill two criteria--the ability to bind to the EpRE and interact with the ER (ref. 76). GST assays were used to confirm that ER-beta interacts with hPMC2 (figure 7a). Binding of hPMC2 and ER-beta to the EpRE was verified using in vitro gel shift assays using purified hPMC2 (figures 7b and 7c). Recruitment of ER-beta and hPMC2 to the EpRE was also demonstrated using Chromatin Immunoprecipitation (ChIP) assays (figure 7D). The binding of hPMC2 to the EpRE induced an increase in EpRE enhancer activity (figure 8). While only a slight upregulation of NQO1 gene transcriptional activity was observed with hPMC2, antiestrogen-ligated ER-beta enhanced hPMC2-mediated activation of NQO1 transcriptional activity (figure 8).

hPMC2 is the human homolog of XPMC2, which was originally cloned from X. Laevis as a gene involved in cell cycle regulation (77). XPMC2 prevents mitotic catastrophe in yeast resulting from disruption of Cdc2 kinase regulation of Wee1 and Mik1 kinase activities (77). Control of Cdc2 kinase is a key step in controlling cell cycle transition from G1 to S phase and G2 to M (78). Wee1 and Mik1 kinase negatively regulate Cdc2 kinases by phosphorylating a conserved tyrosine residue (79). XPMC2 was found to be a substrate for Cdc2 kinase, and XPMC2 acts as a negative cell cycle regulator by competing with mitotic substrates for phosphorylation by Cdc2 kinase (77). The target of XPMC2 action is unknown. hPMC2 may act as a cell cycle regulator by its regulation of NQO1 transcriptional activity. Increased levels of reactive oxygen species affects the efferent pathways involved in cell cycle control, leading to p21 induction (80). p21 induction has been correlated with a suppression of the activity of Cdc2 and other CDKs associated with cyclin A.

Our findings support a model for ER-mediated regulation of NQO1 transcriptional activity that involves the interaction of the ER with activators of EpRE activity. There is a good concordance between the ER isoform that interacts with hPMC2 and the ER isoform that induced NQO1 transcriptional activity. However, our studies also indicate that the interaction of hPMC2 with ER-beta and enhancement of hPMC2 binding to the EpRE may not be the only prerequisite for activation. While antiestrogen-ligated ER-beta interacted strongly with hPMC2 and enhanced hPMC2 binding to EpRE, so did unleaded ER-beta. However unleaded ER-beta did not enhance hPMC2 induction of NQO1 transcriptional activity. Similarly DNA binding by the ER alone is not sufficient for full transcriptional activation and requires interaction with other coregulator factors (81). It is possible that enhancement of hPMC2 activity by ER-beta involves other factors in addition to the ER and hPMC2. Our observations that estrogen-ligated ER-beta only exhibits weak interaction with hPMC2 and did not enhance hPMC2-mediated induction of EpRE enhancer activity suggest another intriguing possibility. It is possible that the increase in NQO1 transcriptional activity may not only result from transcriptional activation by antiestrogen-ligated ER but the release from repressive effects of estrogen-ligated ER. Increased NQO1 transcriptional activity in the presence of...
3.5. Antiestrogens upregulate the transcriptional activity of other genes involved in antioxidative response

Because the EpRE has been identified in the 5′ regulatory region of other antioxidative stress enzymes, it is possible that ER is involved in the regulation of expression of the genes encoding these other enzymes as well. In addition to NQO1 we observe upregulation of the protein levels of two other antioxidative enzymes, GST-Pi and GCSH in the presence of antiestrogens (figure 9A, ref. 82).

We have determined that the EpRE is required for ER-mediated transactivation of the GST-Pi gene promoter (figure 9B and 9C). The ER also regulates GCSH gene transcriptional activity and this activation is mediated by the EpRE and ER-beta as well (figure 9D and 9E). Another
group has reported that tamoxifen induces an increase in the mRNA levels of other phase II detoxification enzymes in rat liver (83). The induction of NQO1 gene transcriptional activity by antiestrogens was also evident in more than one cell context (2, 74). These findings raise the intriguing possibility that antihormones might regulate, in several cellular contexts, the activity of numerous proteins that contain EpREs in their regulatory regions, and thereby afford substantial chemoprotective benefit to ER-containing cells.

4. FUNCTIONAL IMPLICATIONS OF ANTIESTROGEN REGULATION OF NQO1: PROTECTION AGAINST ESTROGEN-INDUCED DNA DAMAGE

4.1. Physiological levels of E2 induce DNA damage independent of ER and dependent on E2 metabolism

It has been reported that metabolites of estrogen, termed catecholestrogens, can form DNA adducts and cause oxidative DNA damage (50-52). We hypothesized that NQO1 inhibits estrogen-induced DNA damage by detoxification of reactive catecholestrogens. We used 8-hydroxydeoxyguanine (8-OHdG) as a marker for oxidative damage because it is one of the most common oxidized bases and has demonstrated mutagenic potential (84). 8-OHdG lesions result in mutational frequencies of 1-5% (mainly G:C to T:A transitions) (85), and may also be diagnostic in that both normal and malignant breast tissue from breast cancer patients was shown to have higher levels of 8-OHdG than control subjects (86, 87). Although previous studies have shown estrogen-induced oxidative DNA damage in cultured breast cells, typically high concentrations of estrogen have been used (88, 89). Also, these studies have used in vitro methods such as HPLC-EC to determine oxidative damage to DNA that have produced controversial results (84). Thus, to determine the effect of physiological concentrations of estrogen, we treated breast cells with physiological doses of E2 (10^{-10} M - 10^{-8} M) and then measured the oxidative DNA marker 8-OHdG by quantitative immunocytochemistry (90). This is a previously established method for quantifying 8-OHdG levels in cells (91) and unlike methods that involve prior isolation of DNA, this method does not create artificial oxidative modification during the procedure. This method also allowed us to quantify 8-OHdG immunoreactivity per cell rather than total 8-OHdG of a cell population.

We observed that physiological concentrations of E2 cause oxidative DNA damage (as measured by levels of 8-hydroxydeoxyguanine) in ER positive MCF7 breast cancer cells, MDA-MB-231 breast cancer cells (ER-alpha negative/ER-beta positive) and nontumorigenic MCF10A breast epithelial cells (very low ER) (figure 10). As a control, we combined E2 with the antioxidant N-acetylcysteine (NAC). As expected, NAC reduced the 8-OHdG induced by E2 (figure 10A). NAC alone had no effect on basal levels.

While the increase in 8-OHdG is independent of ER-mediated proliferation, our results suggest that it is dependent upon estrogen metabolism. Treating the cells with the estrogen metabolism inhibitor alpha-naphthoflavone (CYP1A and 1B) blocked the E2 effect (figure 11A). alpha-Naphthoflavone or its solvent DMSO had no effects on basal levels of 8-OHdG. In breast epithelial cells, E2 is metabolized to the hydroxylated catechols, with the formation of the more carcinogenic 4-OHE2 being favored over 2-OHE2 (53, 58). Consistent with this, we see increased 8-OH-dG only with 4-OHE2 (figure 11B).
To determine the functional significance of antiestrogen-mediated induction of NQO1, we monitored the effect of antiestrogens and NQO1 on estrogen-induced oxidative damage, as NQO1 may be able to detoxify the reactive catecholestrogen quinones (50-52). We see inhibition of E2-induced 8-OHdG with pharmacological concentrations of TOT and ICI 182,780 but only in cells containing high ER, confirming a probable ER-mediated effect (figure 12).
Figure 10. Estrogen induces ODD in breast epithelial cells (A) MCF7 breast cancer cells were grown on coverslips and treated with vehicle (control) or increasing doses of E₂ for 24 hr. As a control for oxidative damage, cells were treated with NAC (300 μM) ± E₂. The cells were then immunostained for 8-OHdG using the 1F7 monoclonal antibody (1:100; Trevigen, Gaithersburg, MD). Shown are the relative levels of 8-OHdG after quantification. Values are the means ± S.E. of three adjacent fields from three or more separate experiments. *denotes level of significance p=0.01 versus control and ** denotes level of significance p≤1x10-6 versus control as determined by t-Test. (B) Same experiments using MCF10A or MDA-MB-231N cells treated with E₂ (10-8 M). ** denotes p≤0.001, * denotes p≤ 0.005 versus control as determined by t-Test. Reprinted by permission from the Endocrine Society (90).
Figure 11. Estrogen induced ODD is dependent on estrogen metabolism. (A) MCF7 cells were grown on coverslips and treated for 24 hr with $10^{-8}$ M E$_2$ ± α-naphthoflavone (5 μM in DMSO) and stained for 8-OHdG. As controls, cells were treated with the vehicles ethanol (control) or DMSO. Shown are the relative levels of 8-OHdG after quantification. * denotes level of significance $p<0.00001$ versus control as determined by t-Test. (B) MCF7 cells grown on coverslips were treated with vehicle (control) or indicated concentrations of 2-OHE$_2$ or 4-OHE$_2$ for 24hr and immunostained for 8-OHdG. Shown are the relative levels of 8-OHdG after quantification. Values are the means ± S.E. of three adjacent fields from two or more separate experiments. * denotes level of significance $p<0.01$ versus control and ** denotes level of significance $p<1x10^{-6}$ versus control as determined by t-Test. Reprinted by permission from the Endocrine Society (90).
Figure 12. Antiestrogens protect against estrogen induced ODD in MCF7 cells, not MCF10A cells. MCF7 (A) or MCF10A (B) cells grown on coverslips were treated with vehicle (control) or E$_2$ (10$^{-8}$ M) ± the antiestrogens (AE) trans-hydroxytamoxifen (TOT, 10$^{-7}$M), ICI-182,780 (ICI, 10$^{-7}$ M), or Raloxifene (RAL, 10$^{-7}$M) for 24hr and immunostained for 8-OHdG. Shown are the relative levels of 8-OHdG after quantification. Values are the means ± S.E. of three adjacent fields from two or more separate experiments. * denotes level of significance p<0.05 versus E$_2$ alone as determined by t-Test. Reprinted by permission from the Endocrine Society (90).
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4.2. Role of NQO1 and ER-beta in antiestrogen inhibition of estrogen-induced DNA damage

To determine whether NQO1 and ER-beta can protect against E$_2$-induced 8-OHdG, we transiently over- or under-expressed NQO1 and ER-beta in MCF7 cells using the self-contained tetracycline-regulated pBPSTR1 vector (92) containing sense or antisense NQO1 cDNA or ER-beta cDNA respectively (90).

Simply overexpressing NQO1 inhibited E$_2$-induced 8-OHdG (figure 13). In contrast, reduction of NQO1 activity by antisense inhibition leads to an increase in control and E$_2$-induced 8-OHdG levels. However, 8-OHdG was not elevated in TOT-treated NQO1AS cells, perhaps due to compensation by TOT-induced NQO1 gene transcription. Further support for this hypothesis comes from the fact that MCF10A cells were still susceptible to damage after TOT treatment perhaps due to very low ER, and thus would not be able to compensate. Decrease in ER-beta expression lead to elevated levels of 8-OHdG in MCF7 cells, and TOT was significantly less protective against E$_2$-induced 8-OHdG (figure 14). However, we cannot say for certain that the protective effect of tamoxifen is through NQO1 alone or other detoxification genes that may also be regulated by ER at the EpRE.

Finally, we measured levels of the DNA repair enzyme, Xeroderma Pigmentosum Group A (XPA) that is involved in nucleotide excision repair of bulky nucleotide lesions and oxidative DNA damage such as 8-OHdG (93-98). Thus, XPA may be involved in the repair of both oxidative damage and/or DNA adducts caused by estrogen. We observed an increase in XPA in the presence of E$_2$ that correlates with levels of 8-OHdG (figure 15A), suggesting that the cells are responding by increased repair. We also found a significant rise in XPA in cells with decreased ER-beta (figure 15B), suggesting that ER-beta plays a protective role against DNA damage and the resulting induction of repair enzymes. The fact that there was no further increase in XPA with E$_2$ in ER-betaAS cells relative to untreated cells suggests to us that there is already a maximal induction of XPA in these cells. Although the induction of XPA is rather modest, they are similar to levels of XPA induced after 20 hr treatment with cisplatin in an ovarian carcinoma cell line (99).

In summary the levels of 8-hydroxydeoxyguanine were directly correlated to levels of the DNA repair enzyme XPA and inversely correlated to NQO1 and ER-beta levels. Trans-hydroxytamoxifen (TOT) and the pure antiestrogen ICI-182,780 protected against E$_2$-mediated damage in MCF7 cells containing ER-beta. This is most likely due to the ability of these antiestrogens to activate expression of NQO1 via ER-beta. It is important to note that E$_2$-induced increase in 8-OHdG was not dependent on ER-mediated proliferation, as both MCF10A and MDA-MB-231 cells also incurred damage, neither of which proliferate in response to E$_2$ (100-101). Also by using immunocytochemistry, we were able to quantify 8-OHdG immunoreactivity per cell rather than total 8-OHdG of a cell population. Although tamoxifen did not induce damage in ER containing MCF7 cells, there was a modest but significant

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Figure 13. Quinone reductase protects against estrogen induced ODD. (A) MCF7 cells were transiently infected with QR retroviruses or control retroviruses. The cells were then treated with vehicle (control), E$_2$ ($10^{-8}$ M), or the antiestrogen trans-hydroxytamoxifen (TOT, $10^{-7}$ M) for 24 hr and immunostained for 8-OHdG. * denotes level of significance $p<0.01$ versus vehicle treated control cells as determined by t-Test. (B) Same experiment except MCF7 cells were transiently infected with QRAS retroviruses or control retroviruses. a denotes level of significance $p\leq 0.01$ versus respective control as determined by t-Test. b denotes level of significance $p\leq 0.05$ versus cells infected with control retroviruses with same treatment as determined by t-Test. (C) MCF10A cells were transiently infected with QRAS retroviruses or control retroviruses. * denotes level of significance $p<0.01$ versus cells infected with control retroviruses with same treatment as determined by t-Test. Shown are the relative levels of 8-OHdG after quantification. Values are the means ± S.E. of three adjacent fields from two or more separate experiments. Reprinted by permission from the Endocrine Society (90).
induction of 8-OHdG in MCF10A cells. It is possible that tamoxifen causes oxidative damage in ER negative cells, while being protective in ER positive cells through induction of detoxification enzymes such as NQO1. Differences in the way tamoxifen is metabolized, in conjugation of tamoxifen metabolites by Phase II enzymes, or in DNA repair mechanisms can also bring the differential induction of DNA damage by tamoxifen. While the chemical structure of ICI182,780 would predict increased 8-OH-dG from its metabolism, there has been no reports of induction of oxidative DNA damage by ICI182,780.

5. PERSPECTIVE

We have shown that NQO1, a detoxifying enzyme, is markedly induced by antiestrogens in breast cancer cells. Antiestrogens induce an increase in NQO1 mRNA in breast cancer cells, and our studies indicate that the regulation of NQO1 activity by antiestrogens occurs at the transcriptional level and is mediated by the ER and an EpRE element in the NQO1 gene. Gel shift and ChIP analyses reveal that the ER can bind to the EpRE. Activation by antiestrogens may be modulated by differences in the relative levels of ER-alpha and ER-beta in different target cells. Our characterization of hPM2C and its role in NQO1 transcriptional regulation indicate that antiestrogen-mediated increase in NQO1 activity may also involve antiestrogen-ER enhancing the activity of other factors that interact with the EpRE. The NQO1 gene is unusual in that it is an antiestrogen-stimulated gene and shows "reversed pharmacology", being induced in ER-containing cells by antiestrogen treatment and suppressed when the ER is occupied with estrogen.

Our findings indicate that antiestrogens regulate the activity of other antioxidative enzymes that contain EpREs in their regulatory regions, and thereby afford substantial chemoprotective benefit to ER-containing cells. Although signaling pathways that regulate NQO1 activity eventually converge and act through the EpRE, by studying the mechanism of ER-dependent activation of NQO1 by antiestrogens we can gain insight into other relevant pathways that may be different from the electrophile-dependent pathways utilized by more well-known inducers of NQO1.

The findings from these studies will provide new insight into how antiestrogens function as anticancer drugs,
Figure 15. Estrogen and decreased levels of ER-βeta lead to elevated XPA levels in breast epithelial cells. (A) MCF7 or MCF10A cells grown on coverslips were treated with vehicle (control), E2 (10^{-8} M), or the antiestrogen trans-hydroxytamoxifen (TOT, 10^{-7} M) for 24hr. The cells were then immunostained for XPA and quantified. Values are the means ± S.E. of three adjacent fields from three or more separate experiments. * denotes level of significance p≤0.05 versus respective vehicle treated cells as determined by t-Test. (B) MCF7 cells were transiently infected with ER-βetaS retroviruses, ER-βetaAS retroviruses or control retroviruses. The cells were then treated with vehicle (control), E2 (10^{-8} M), and/or the antiestrogen trans-hydroxytamoxifen (TOT, 10^{-7} M) for 24 hr and immunostained for 8-OHdG. Shown are the relative levels of 8-OHdG after quantification. Values are the means ± S.E. of three adjacent fields from two or more separate experiments. a denotes level of significance p<0.05 versus respective vehicle treated cells; b denotes p≤0.05 versus E2 alone; c denotes level of significance p<0.05 versus cells infected with control retroviruses with same treatment as determined by t-Test. Reprinted by permission from the Endocrine Society (90).
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which may derive not only from the already well-known repression of estrogen-stimulated activities, but also from the activation of detoxifying enzymes. NQO1 reduces the secondary toxicity of certain cytotoxic agents that are mediated by semiquinone redox cycling. Indeed the metabolism of estrogens can be one of these processes that induce genotoxic effects. We observed that upregulation of NQO1, either by overexpression or induction by TOT, can protect breast cells against oxidative DNA damage caused by estrogen metabolites, representing a possible novel mechanism of tamoxifen prevention against breast cancer. It remains to be examined if NQO1 and ER can also inhibit another type of DNA damage, DNA adducts formed by reactive catechol estrogens.

The activation of NQO1 activity by antiestrogens may thus contribute to the beneficial antioxidant activity of antiestrogens in breast cancer and possibly other estrogen target tissues. The ability of antiestrogens such as tamoxifen to increase NQO1 levels and activity also has important potential implications for increasing the potency of certain chemotherapeutic agents that are activated by quinone reduction. Although regulation of the effectiveness of cancer chemotherapeutic agents is complex, antiestrogen treatment might also enhance the sensitivity of tumor cells to agents that are activated by quinone reduction, such as mitomycin C and aziridylbenzoquinones (26-30). This may contribute to the beneficial effects of antiestrogens in cancer therapy and in chemoprevention. Antiestrogens like tamoxifen are being employed extensively in both therapeutic trials and in chemoprevention trials; thus studies on possible effects of antiestrogens on the relative efficacy of chemotherapeutic agents are of clinical relevance. By examining the effect of antiestrogens on the sensitivity of breast cancer cells to bioreductive chemotherapeutic agents that are activated by NQO1, these studies may contribute toward a more effective selection of adjuvant chemotherapy for the treatment of breast cancer.

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