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TARGETING THE GENOTOXIC EFFECTS OF ESTROGENS

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Abstract

Our studies indicate that expression of antioxidative stress enzymes is upregulated by Selective Estrogen Receptor Modulators (SERMs) in breast epithelial cell lines, providing protection against the genotoxic effects of estrogens and against estrogen-induced mammary tumorigenesis. This upregulation of antioxidative stress enzymes requires Estrogen Receptor beta (ER β) and human homolog of *Xenopus* gene which Prevents Mitotic Catastrophe (hPMC2). Further studies indicate that hPMC2 has a functional exonuclease domain that is required for upregulation of antioxidative stress enzymes by SERMs and repair of estrogen-induced abasic sites.

Introduction

Lifetime exposure to estrogen is a major risk factor for breast cancer. It has been proposed that tumor initiation is not due to ER-mediated proliferation, but rather DNA damage due to a combination of estrogen metabolism and preexisting lesions [1]. After tumor initiation, ER confers a selective advantage to these premalignant cells. The genotoxic effects of estrogen metabolites and their role in breast tumor initiation have been a controversial subject. There is concern that 17 β -Estradiol (E $_2$) levels are expected to be low in breast tissue based on plasma uptake [2]. However, tissue concentrations do not always correlate to plasma levels due to intracellular synthesis through the aromatase enzyme [2]. Moreover, there are significant levels of the more carcinogenic metabolites of E $_2$, 4-OHE $_2$, in rat mammary tumor tissues and human breast cancer tissues [3]. While the role of estrogen metabolites in breast cancer initiation or development has been extensively studied, there is less work on the inhibition of this process.

Breast cancer chemoprevention

In 1998, tamoxifen became the first drug to be approved for the reduction of risk for breast cancer. Raloxifene has also been shown to reduce the risk of invasive breast cancer in women with osteoporosis and is as effective as tamoxifen for reducing the risk of invasive breast cancer in postmenopausal women [4]. Studies described in this review provide insight as to how lifetime exposure to estrogens increases breast cancer risk and how SERMs prevent cancer, and thus are important for developing new drugs. It was proposed that aromatase inhibitors (AI) may be more effective because they exert dual effects on proliferation and genotoxic metabolite formation than SERMs, which can only block ER-

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mediated proliferation [5]. Our findings that tamoxifen and raloxifene inhibit estrogen-induced DNA damage by upregulating expression of antioxidative stress enzymes [6] argue against the idea that SERMs only block ER-mediated proliferation. Since AI use is associated with menopausal symptoms, an alternative treatment for breast cancer prevention ideally would include a medication that prevents estrogen action in the breast but does not require that estrogen be deficient.

Role of Estrogen Receptor β in mammary tumorigenesis

Some studies correlating ER β protein expression with breast tumor grade predict good prognosis for ER β positive tumors [7–11]. ER β [7,12]. Because ER β has anti-growth properties, selective activation of ER β in cells may suppress growth of estrogen-dependent tumors. ER β has also been reported to act as a dominant negative regulator of ER α -mediated transcription, thus attenuating estrogenic stimulation [13–16]. Expression of ER β protein in *BRCA1*-associated tumors would explain the protective effects of antiestrogens [17]. TAS-108, a SERM showing antagonistic properties toward ER α signaling accompanied with a partial agonistic effect for ER β , is undergoing phase II trials for metastatic breast cancer treatment [18]. Characterization of the mechanism of ER β -mediated regulation of antioxidative enzymes has important implications for the role of ER β in breast cancer.

Identification of hPMC2 as a protein factor that interacts with ER β and regulates expression of genes encoding antioxidative stress enzymes

The electrophile response element (EpRE), also referred to as the antioxidant responsive element (ARE), is found in the 5'-regulatory region of a number of genes encoding phase II, drug-metabolizing enzymes. We used yeast genetic screenings to identify hPMC2 (human homolog of *Xenopus* gene which Prevents Mitotic Catastrophe) as a factor that binds to the EpRE and interacts with the ER in yeast genetic screening and *in vitro* assays [19]. We determined the extent to which hPMC2, by mediating ER β transactivation, regulates not only the antioxidative response but also the anti-growth properties of ER β .

To determine the mechanism for ER β -hPMC2 activation of EpRE enhancer activity, we examined the ability of ER β and hPMC2 to recruit ER-associated coactivators and induce expression of antioxidative genes in the absence of ER α . Factors typically involved in DNA damage/repair machinery also appear necessary for ER-mediated transactivation. TopoII relaxes DNA strands and favors chromatin bending to accommodate the transcription initiation complex [20]. DNA strand breaks (DSBs) created by TopoII then trigger PARP-1 catalytic activity, poly(ADP-ribosyl)ation of chromatin-associated proteins, and release of PARP-1 corepressor complex [20]. We observed trans-hydroxytamoxifen (TOT)-dependent recruitment of Nrf2, ER β , hPMC2, PARP-1, TopoII and SRC-1, and dependence on both ER β and hPMC2 for TOT-mediated recruitment of the coactivator complex at the EpRE.

A report by Lee et al. indicated that Nrf2, not ER, mediates activation of the EpRE by catechol estrogen in a neuroblastoma cell line and primary astrocytes [21]. Kim et. al. also reported that Nrf2, not ER, plays a role in regulation of gamma-glutamylcysteine ligase heavy chain (gamma-GCLh), an antioxidant protein also regulated through the EpRE [22]. In both cases, the authors utilized micromolar concentrations of 4-OHE₂, E₂, or TOT. At these levels estrogens and tamoxifen would not be expected to function primarily through the ER. Moreover, experiments were not performed in ER negative cells or in ER positive cell lines after downregulation of ER to unequivocally show that ER is not involved in this regulation. Independent observations of another group support our findings of tamoxifen-ER-mediated upregulation of antioxidative stress enzymes in breast epithelial cells and ER-transfected COS1 cells [23].

The exonuclease of hPMC2 is required for upregulation of antioxidative stress enzymes

The C-terminus of hPMC2 encodes a putative exonuclease domain. Proteins that possess exonuclease domains are involved in a wide variety of cellular functions and have important roles in repair of DNA breaks, recruitment of DNA damage/repair factors, as well as in transcriptional regulation [24]. We thus determined whether the exonuclease activity played a role in its ability to regulate transcription of genes encoding antioxidative stress enzymes. The 3'→5' exonuclease activity of hPMC2 was demonstrated by examining product formation from 5' vs. 3' labeled substrates [25]. Endonuclease activity was demonstrated using a circular plasmid substrate. The catalytic activity of hPMC2 appears to be important for its ability to upregulate quinone reductase (QR) expression. We then determined whether TOT could induce DSBs in the EpRE region of the QR promoter and the role of hPMC2 exonuclease activity in this process. DSBs were detected via biotin-11-deoxyuridine triphosphate (biotin-dUTP) labeling by terminal deoxynucleotide transferase, and ChIP assays performed using anti-biotin antibodies [20]. We observed that TOT-induced DSBs on the EpRE-, but not ERE-, containing regions, and that hPMC2 exonuclease activity is necessary for DSB formation. The DSB formation, in turn, creates a signal resulting in activation of PARP-1 and ultimately serves as a mechanism for initiation of gene transcription. Thus, our data mechanistically links the exonuclease activity of hPMC2 to components of DNA damage and repair machinery in the regulation of gene transcription.

Functional implications of SERM regulation of antioxidative stress enzymes: protection against estrogen-induced DNA damage

It has been reported that metabolites of estrogen, termed catecholestrogen quinones, can form DNA adducts and cause oxidative DNA damage (ODD, [2,3,26]). QR may inhibit estrogen-induced DNA damage by detoxification of reactive derivatives of catecholestrogens. Data from *in vitro* studies from other laboratories suggest that estrogen quinones are not substrates for QR [27,28]. While their conclusion was based on indirect evidence, the laboratory of Drs. Cavalieri and Rogan demonstrated that 4-OHE₂ is produced from E₂-3,4-quinone in the presence of recombinant QR [29]. We also showed that downregulated QR expression in MCF10A cells resulted in accumulation of estrogen quinone conjugates [30] and of depurinating adducts after E₂ treatment, further supporting that estrogen quinones are substrates for QR. Work from Fritz Parl's group also supports estrogen quinones being substrates for GSTpi [31,32].

We used 8-OHdG as a marker for oxidative damage because it is one of the most common oxidized bases and has demonstrated mutagenic potential [33]. 8-OHdG lesions may be prognostic in that both normal and malignant breast tissue from breast cancer patients was shown to have higher levels of 8-OHdG than control subjects [34,35]. Although previous studies show estrogen-induced ODD in cultured breast cells, typically high concentrations of estrogen have been used [36]. These studies used *in vitro* methods such as HPLC-EC to determine oxidative damage to DNA that have produced controversial results [37]. We measured the oxidative DNA marker 8-OHdG by quantitative immunocytochemistry [6]. We observed that physiological concentrations of E₂ caused ODD in ER positive MCF7 breast cancer cells, MDAMB-231 breast cancer cells (ER negative/ ER (ER negative/ ER [6]. The antioxidant N-acetylcysteine (NAC) reduced ODD induced by E₂, while having no effect on basal levels [6]. It is important to note that the E₂-induced increase in 8-OHdG and MCF10A cell transformation were not dependent on ER-mediated proliferation, as both MCF10A and MDA-MB-231 cells also incurred damage, neither of which proliferate in response to E₂ [38,39]. Using immunocytochemistry, we were also able to

quantify 8-OHdG immunoreactivity per cell rather than total 8-OHdG of a cell population. While the increase in 8-OHdG is independent of ER-mediated proliferation, our results suggest that it is dependent upon estrogen metabolism.

ER β and hPMC2 are required for effective inhibition of estrogen-induced oxidative DNA damage by tamoxifen

We also measured the effect of E₂ treatment on the levels of catechol estrogens and their quinone-conjugates in the absence or presence of TOT. We measured the levels of E₂-3,4-quinone conjugate (4-con), as this metabolite is predominantly responsible for the formation of mutagenic DNA adducts [40]. TOT treatment reduced both 4- and E₂-2,3-quinone conjugate levels. The TOT-dependent decrease in the levels of E₂-induced quinone-conjugates is dependent on the presence of ER and hPMC2 and not on the levels of the conjugates themselves.

The catalytic activity of hPMC2 is required for removal of estrogen-induced abasic sites

As part of their proofreading activity, 3'→5' exonuclease domains can catalyze the removal of abasic sites [41], such as those that result from estrogen-induced ODD and DNA adduct formation [42, 43]. The apurinic/apyrimidinic (AP) sites resulting from the spontaneous depurination/depyrimidination of modified bases and from the oxidative damage to the deoxyribose moiety of DNA molecules will lead to aldehydic forms of DNA lesions [42,43]. If not repaired, AP sites are promutagenic DNA lesions. Thus, it would be of interest if hPMC2 attenuates estrogen-induced DNA damage by playing a more direct active role in its repair. It is possible that the hPMC2 exonuclease activity is separate from its ability to regulate transcription. HAP1 protein, the human homologue of *Escherichia coli* exonuclease III protein, not only possesses DNA repair activity but also plays a role in transcription by redox regulation of Jun DNA binding activity [44]. Similarly p53 has transactivation functions and an exonuclease activity that may be involved in DNA repair [45].

We used a biotinylated hydroxylamine Aldehyde-Reactive Probe (ARP, Molecular Probes, Eugene, OR) to detect abasic sites. ARP modifies the exposed aldehyde group in an abasic site, and the resulting biotinylated DNA can be quantitated using fluorescent or enzyme-conjugated streptavidin complexes. Our results revealed that in control MCF10A cells, treatment with E₂ resulted in a 3-fold increase in the number of AP sites, indicating that estrogen causes oxidative DNA damage or formation of depurinating estrogen-DNA adducts and that the exonuclease activity of hPMC2 is required for the repair of estrogen-induced abasic sites in DNA. While the ability of hPMC2 to repair DNA may be of relevance in its role in cancer prevention, hPMC2 may attenuate the ability of cancer cells to undergo apoptosis and desensitization to chemotherapeutic agents. We are currently testing the ability of hPMC2 to modulate responses to other cancer chemotherapeutic agents.

Induction of quinone reductase by tamoxifen or an ER β agonist protects against mammary tumorigenesis

We have previously shown that ER-mediated upregulation of QR is involved in the protection against estrogen-induced mammary tumorigenesis in the ACI rat [30]. Studies in Aromatase (Arom) transgenic models have shown that local estrogen is directly involved in the initiation of preneoplastic changes in the mammary epithelium [46]. Overexpression of aromatase in the mammary glands of Arom mice leads to hyperplasia and changes in the expression of genes involved in apoptosis, cell cycle and tumor suppression functions [46].

Our studies indicate that the ER agonist, 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN) and the SERM tamoxifen (Tam), inhibit estrogen-induced DNA damage and mammary tumorigenesis in the aromatase transgenic (Arom) mouse model [47]. We also show that either DPN or Tam treatment increases QR levels and results in a decrease in ductal hyperplasia, proliferation, ODD, and an increase in apoptosis. In order to corroborate the role of QR, we provide additional evidence in triple transgenic MMTV/QR/Arom (AQM) mice wherein the QR expression is induced in the mammary glands via doxycycline, causing a decrease in ductal hyperplasia and ODD. Overall, we provide evidence that upregulation of QR through induction by Tam or DPN can inhibit estrogen-induced ODD and mammary cell tumorigenesis, representing a novel mechanism of prevention against breast cancer. Our data thus have important clinical implications in the management of breast cancer and brings forth potentially new therapeutic strategies involving ER agonists.

It has been reported that tamoxifen can form quinone metabolites in vitro and have genotoxic effects [48]. However, these studies indicate genotoxic effects of tamoxifen at 300-fold higher levels than the concentration of tamoxifen we have shown to be required for tamoxifen protection against the genotoxic effects of estrogens. It should be noted that the efficacy of lower doses of tamoxifen is similar to that seen with a standard dose of the drug, and there has been a reduction in healthcare costs and side effects [49–51]. Although the therapeutic dose of 20 mg/day tamoxifen has been used in all the phase III chemoprevention trials, there is compelling evidence that a lower dose, e.g., 5 mg/day or even less, could have the same protective effect while also lowering the drug's estrogenic effects, thereby potentially reducing the risks of endometrial cancer and venous thromboembolic events [51].

We are not discounting other ways that tamoxifen can have chemopreventive effects. Tamoxifen activates signal transduction kinases [52] that have also been shown to regulate EpRE enhancer activity [53]. However the ER may not be necessary for these tamoxifen-mediated responses, while tamoxifen regulation of antioxidative enzymes requires ER.

Conclusion

Our studies provide novel insights into protein activity or pathways that may be disrupted and allow enhanced tumorigenic effects of estrogens. These studies should also aid in the design of new therapies targeting direct mutagenic effects of estrogens.

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