Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: potential biomarkers of susceptibility to cancer

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Exposure to estrogens has been associated with an increased risk of developing breast cancer. Breast biopsy tissues from 49 women without breast cancer (controls) and 28 with breast carcinoma (cases) were analyzed by HPLC with electrochemical detection for 31 estrogen metabolites and catechol estrogen quinone-glutathione conjugates. The levels of estrone and estradiol were higher in controls, suggesting in the cases a higher probability for mutations that may initiate cancer. The levels of 4-CE were nearly four times higher in cases than in controls. Less O-methylation was observed for the CE in cases. The level of catechol estrogen quinone conjugates in cases was three times higher than in controls, but the 4-CE were three times higher than in controls. In addition, the 4-CE were nearly four times higher in cases than in controls. Less O-methylation was observed for the CE in cases. The level of catechol estrogen quinone conjugates in cases was three times higher than in controls, suggesting in the cases a higher probability for the quinones to react with DNA and generate mutations that may initiate cancer. The levels of 4-CE and quinone conjugates were highly significant predictors of breast cancer. These results suggest that some catechol estrogen metabolites and conjugates could serve as biomarkers to predict risk of breast cancer.

Introduction

Estrogens have been implicated in the etiology of human breast cancer by various types of evidence (1–6). They are generally thought to cause proliferation of breast epithelial cells through estrogen receptor-mediated processes (3). Rapidly proliferating cells are susceptible to genetic errors during DNA replication, which, if uncorrected, can ultimately lead to malignancy. While receptor-mediated processes may play an important role in the development and growth of tumors, accumulating evidence suggests that specific oxidative metabolites of estrogens, if formed, can be endogenous ultimate carcinogens that react with DNA to cause the mutations leading to initiation of cancer (5–7).

Studies have proposed that induction of breast cancer was caused by a covalent bond of 16α-hydroxysterone (16α-OHE1), a metabolite of E1, with the estradiol (E2) receptor. This receptor modification would result in a permanent, uncontrolled stimulation of cell proliferation by receptor-mediated processes (8–10). This hypothesis implies a correlation of high levels of 16α-OHE1 with induction of breast cancer. Over the years, however, this hypothesis has never been substantiated.

Several lines of evidence, including metabolism and carcinogenicity studies by Liehr et al., led to the recognition that the 4-hydroxylated estrogens play a major role in the genotoxic properties of estrogens (1, 2, 11). We have hypothesized that estrogens, E1 and E2, initiate breast cancer by reaction of their electrophilic metabolites, catechol estrogen-3,4-quinones [E1(E2)-3,4-Q], with DNA to form depurinating adducts (4–6). These adducts generate apurinic sites leading to mutations that may initiate breast, prostate and other human cancers (5–7). The estrogens, E1 and E2, are obtained via aromatization of 4-androstene-3,17-dione and testosterone, respectively, catalyzed by cytochrome P450(CYP19), aromatase (Figure 1). E1 and E2, which are biochemically interconvertible by the enzyme 17β-estradiol dehydrogenase, are metabolized to the 2-catechol estrogens (CE), 2-OHE1(E2) and 4-OHE1(E2), predominantly catalyzed by the activating enzymes CYP1A1 (12) and 1B1 (12–15), respectively, in extrahepatic tissues. The estrogens are also metabolized, to a lesser extent, by 16α-hydroxylation (not shown). The CE are further oxidized to the E1(E2)-2,3-Q and E1(E2)-3,4-Q (Figure 1). In general, the CE are inactivated by conjugating reactions, such as glucuronidation and sulfation. A common pathway of inactivation in extrahepatic tissues, however, occurs by O-methylation catalyzed by the ubiquitous catechol-O-methyltransferase (COMT) (16). If formation of E1 or E2 is excessive, due to overexpression of aromatase and/or the presence of excess sulfatase that converts the stored E1 sulfates to E1, increased formation of CE is expected. In particular, the presence and/or induction of CYP1B1 and other 4-hydroxylases could render the 4-OHE1(E2), which are usually minor metabolites, as the major metabolites. Thus, conjugation of 4-OHE1(E2) via methylation in extrahepatic tissues might become insufficient, and competitive catalytic oxidation of 4-OHE1(E2) to E1(E2)-3,4-Q could occur.

Protection at the quinone level can occur by conjugation of CE quinones with glutathione (GSH), catalyzed by S-transferases (Figure 1). A second inactivating process for CE quinones is their reduction to CE by quinone reductase. If these two inactivating processes are not effective, CE quinones may react with DNA to form stable and depurinating adducts (4–6, 17–20).

We hypothesize that imbalances in estrogen homeostasis, that is the equilibrium between activating and protective

Abbreviations: CE, catechol estrogen; Cys, cysteine; GSH, glutathione; NAcCys, 4-OHE1(E2)-2-N-acetylcysteine.

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enzymes with the scope of avoiding formation of CE semi-quinones and quinones, can lead to initiation of cancer by estrogens. In this article we report the identification and quantification of estrogen metabolites and conjugates in human breast tissue, showing statistically significant differences between tissue from women with and without breast carcinoma.

**Materials and methods**

**Subjects**

The subjects for this study were recruited from women undergoing breast biopsies. They included a control group of 49 women subsequently histopathologically diagnosed as having benign breast disease (40 Caucasian, three African-American, two Hispanic and one unknown; age range 31–82, median age 52 years) and a case group of 28 women, 12 subsequently diagnosed with ductal carcinoma in situ and 16 with invasive carcinoma (15 Caucasian, two African-American, two Asian and nine unknown; age range 36–88, median age 54 years). The subjects were recruited under a protocol approved by the Institutional Review Board and informed consent documents are on file.

**Materials**

2-OHE1(E2), 4-OHE1(E2), 2-OCH3E1(E2) and 4-OCH3E1(E2) were synthesized according to Dwivedy et al. (17). 4-OHE1(E2)-2-glutathione (-SG), 4-OHE1(E2)-2-cysteine (Cys), 4-OHE1(E2)-2-N-acetylcysteine (NAcCys), 2-OHE2-1-SG, 2-OHE2-4-SG, 2-OHE2-1&4-SG, 2-OHE1(E2)-1-Cys, 2-OHE1(E2)-1-NAcCys, 2-OHE1(E2)-4-Cys and 2-OHE1(E2)-4-NAcCys were synthesized according to published procedures of Cao et al. (21). 16α-OHE1(E2) and 2-ΟH-3-OCH3E1(E2) were purchased from Steraloids (Newport, RI). E1, E2, all enzymes and chemicals were purchased from Sigma (St Louis, MO). Certify II Sep-Pak cartridges were purchased from Varian (Palo Alto, CA). The Luna(2) HPLC column was purchased from Phenomenex (Torrance, CA).

**Collection and treatment of tissues**

The breast biopsy specimens were submitted directly to the University Hospital gross pathology laboratory from the operating suite within 1 h of excision. The pathologist examined the biopsy specimen upon receipt in the gross laboratory along with the submitted clinical history and available radiographic data for each case. Only excisional biopsies or ABBI (Advanced Breast biopsy Instrument – Imagyn) directed breast biopsies were utilized for this study, as the smaller ‘core’ biopsies did not provide sufficient tissue for both diagnostic purposes and the research protocol. The location and extent of the breast lesion generating the biopsy (mammographic abnormality, palpable abnormality) was identified by the pathologist in conjunction with the surgeon to assure that no lesional tissue or significant marginal tissue was sampled for the research study. An ~1 g sample of grossly unremarkable adipose marginal tissue and normal appearing breast parenchyma was sampled as distant as possible from the lesion of clinical interest, avoiding tumor tissue and marginal tissue. The samples were obtained from areas >3 cm from the lesion of clinical interest. If the breast biopsy was too small to allow this clear separation of the research sample from the lesion, the biopsy was not utilized in the study, and no tissue was submitted for the research protocol. As soon as the research sample was separated from the rest of the biopsy, the research sample was immediately frozen in liquid nitrogen and transferred to a –80°C freezer at the end of the day. The research samples were maintained frozen until at least 2 weeks after the final pathologic diagnosis was rendered, assuring that the sample tissue was not necessary for further pathologic examination.

For analysis, the entire breast biopsy specimen was weighed, partially thawed, minced and ground to a fine powder in liquid nitrogen. Ground tissue was suspended in 2 ml of 100 mM ammonium acetate, pH 4.4, containing 2 mg/ml ascorbic acid, and β-glucuronidase from Helix pomatia (10000 U, also containing 900 U of arylsulfatase) was added and the tissue was incubated for 16 h at 37°C. After incubation, sufficient methanol was added to give a final concentration of 60% by volume, and the mixtures were extracted with 10 ml of hexane to remove any lipids. The methanol extract was diluted with 100 mM ammonium acetate, pH 4.4, containing 1 mg/ml ascorbic acid, to an approximate final concentration of 30% methanol, and the methanol/water mixture was applied to a Certify II Sep-Pak (200 mg) cartridge. The cartridge was first
eluted with 3 ml of the buffer, followed by elutions with 2 ml each of 20, 40 and 70% methanol in buffer, and fractions were collected. To minimize oxidation of the conjugates, ascorbic acid was added to the eluting buffer at a concentration of 1 mg/ml. Collected fractions were analyzed by HPLC with electrochemical detection.

**HPLC analysis**

The analyses, blinded to diagnosis and with the samples in random order, were carried out by using a Luna(2) C18 reverse phase column (250 × 4.6 mm, 5 μm) on an HPLC system equipped with dual ESA Model 580 solvent delivery modules, an ESA Model 540 autosampler and a 12-channel ESA CoulArray electrochemical detector (ESA, Chelmsford, MA). A gradient system was employed for separation of the 31 estrogen metabolites and conjugates of interest. The oxidation potentials were set at −10, 50, 100, 130, 180, 230, 280, 340, 390, 480, 530 and 590 mV, with respect to the internal standard electrode, for channels 1–12. A linear gradient starting from 100% acetonitrile/methanol/water/1 M ammonium acetate, pH 4.4 (15:5:70:10) to 90% acetonitrile/methanol/water/1 M ammonium acetate, pH 4.4 (50:20:20:10) over 50 min was employed to separate the 31 compounds analyzed, at a flow rate of 1 ml/min (Figure 2). The sensitivity of detection for all of the 31 compounds was ~1 pmol (20). Conjugates from the tissue specimens were identified by comparison with authentic standards, based on their retention time, as well as peak height ratios between the dominant peak and the peaks of the E1 and E2 forms of the metabolites and conjugates and report them in Table I and Figures 3 and 4 as combined E1(E2).

**Results**

Women undergo breast biopsies in general because manual or radiographic examination of the breast indicates the possibility of a cancerous growth. The population of women undergoing breast biopsy thus includes women who have only benign breast tissue, including women with fibrocystic breast tissue and women with breast carcinoma. For this study, we included specimens of benign breast tissue from women who showed no sign of malignancy, including those who were diagnosed with fibrocystic breast disease, and women who were diagnosed with breast carcinoma. For the samples from women with carcinoma, the specimen did not include tumor tissue, just grossly benign non-tumorous tissue removed as part of the biopsy. Data from subjects with benign breast diseases were combined to make the control group.

The 77 specimens were analyzed for the levels of E1, E2, their hydroxylated metabolites and both methoxy and GSH conjugates. The GSH conjugates were detected as CE-Cys and CE-NAcCys conjugates because enzymes in the breast cells hydrolyze the GSH moiety to Cys and then acetylate it to NAcCys following the mercapturic acid biosynthesis pathway (22). Since E1 and E2 are continually interconverted by 17β-estradiol dehydrogenase, we added together the amounts of the E1 and E2 forms of the metabolites and conjugates and report them in Table I and Figures 3 and 4 as combined E1(E2). In addition, we added together all of the GSH conjugates detected in each sample and report them as the sum of 4-OHE1(E2)-2-Cys, 4-OHE1(E2)-2-NAcCys, 2-OHE1(E2)-1-Cys, 2-OHE1(E2)-1-NAcCys, 2-OHE1(E2)-4-Cys and 2-OHE1(E2)-4-NAcCys. All of the estrogen compounds were not detected in any one tissue specimen, but every specimen contained at least one compound. Therefore, the means and standard deviations were calculated based on the levels of analytes in the positive specimens, i.e. the specimens in which that particular analyte was detected.

The mean and standard deviation of the positive samples for controls (subjects with benign breast disease) and cases (subjects with breast carcinoma), as well as the number and percentage of positive samples, are reported in Table I. The P-value in Table I compares the estrogen metabolite or conjugate levels (positive samples only) for all controls to cases using the Wilcoxon rank sum test.

Although the mean level of E1(E2) was approximately twice as high in cases as in controls, the difference was not statistically significant. The level of 2-OHE1(E2) was approximately the same in cases and controls (5.4 versus 4.5 pmol/g tissue), but the level of 4-OHE1(E2) was nearly four times higher in the cases than in the controls (13.3 versus 3.4 pmol/g tissue), a statistically significant difference (P = 0.01, Wilcoxon rank sum test). In addition, these metabolites were detected in 54%
of cases and only 10% of controls ($P < 0.001$, Fisher’s Exact test). In all control subjects, the mean level of 2-OHE$_1$(E$_2$) was slightly higher than the level of 4-OHE$_1$(E$_2$) (5.4 versus 3.4), although this comparison did not reach the level of statistical significance. As expected from data reported in the literature (23,24), the ratio of the mean 4-OHE$_1$(E$_2$) to 2-OHE$_1$(E$_2$) in cases versus controls changed dramatically from ~0.6 in controls to ~3 in cases. The percentage of samples in which 2-OHE$_1$(E$_2$) was detected was higher (46%) for the cases than the controls (24%), but the difference was marginally significant ($P \approx 0.08$, Fisher’s Exact test). The levels of 16α-OHE$_1$(E$_2$) (2.8 versus 3.5) showed no difference between the cases and the controls.

Methylation of the 2-OHE$_1$(E$_2$) and 4-OHE$_1$(E$_2$) prevents their further oxidation to CE quinones. Indeed, the levels of 2-methoxyE$_1$(E$_2$) and 4-methoxyE$_1$(E$_2$) were lower in the cases than in the controls, but the differences did not reach statistical significance.

The presence of CE–GSH conjugates, detected as CE-Cys and/or CE-NAcCys conjugates, in breast tissue demonstrates that the CE quinones were present and reacted with GSH. The mean level of CE–GSH conjugates in cases was significantly higher than in the controls (8.2 versus 2.6, $P = 0.003$, Wilcoxon rank sum test). In addition, the number of cases with positive samples was significantly higher than the number of controls (57 versus 29%, $P = 0.02$, Fisher’s Exact test). Thus, we assume that the level of CE quinones is higher in cases than in controls.

**Discussion**

The results obtained in this first study of estrogen metabolites and conjugates in human breast tissue not only provide evidence that imbalances in estrogen metabolism in breast tissue correlate with the development of tumors, but also suggest possible biomarkers related to the risk of developing breast cancer. If, as we have proposed, estrogens initiate breast cancer through formation of depurinating DNA adducts by E$_1$(E$_2$)-3,4-Q, several differences in the profiles of estrogen metabolites and conjugates in women with and without breast cancer can be predicted: (i) breast tissue from women with breast cancer contains more 4-OHE$_1$(E$_2$), lower levels of methoxyCE and higher levels of CE-GSH conjugates; (ii) tissue from women without breast cancer contains more...
2-OHE₁(E₂), higher levels of methoxyCE and lower levels of CE–GSH conjugates. In addition, (iii) the ratio of 4-OHE₁(E₂) to 2-OHE₁(E₂) is greater than 1.0 in women with breast cancer and less than 1.0 in women without.

All of these predictions were borne out by the results of this initial study (Table I). As shown in Figure 3, higher levels of E₁(E₂) (8.0 versus 4.1 pmol/g tissue) were detected in breast tissue from cases compared with the controls; this finding is consistent with concerns about biosynthesis of estrogens in situ as a risk factor in breast cancer and the trial of aromatase inhibitors to prevent development of contralateral breast tumors in women who already have breast cancer (25). Significantly higher levels of 4-OHE₁(E₂) (13.3 versus 3.4 pmol/g tissue, \( P = 0.01 \)) were observed, suggesting that women who develop breast cancer have more CYP1B1 activity in the breast than women without the disease. This finding points toward possible intervention in the synthesis or protection of the 4-OHE₁(E₂). The levels of 2-OHE₁(E₂) were approximately the same in both groups, but the relative amounts of the 4-OHE₁(E₂) and 2-OHE₁(E₂) changed dramatically from a mean ratio of 0.6 in the controls to 3.0 in the cases. The comparable levels of 16α-OHE₁(E₂) in cases and controls was not surprising, as this metabolite cannot be further oxidized and would be expected to play no role in the initiation of breast cancer by estrogen-induced DNA damage. These results contradict the hypothesis formulated by Fishman and Bradlow (8–10), which implies that high levels of 16α-OHE₁ are associated with breast cancer.

Methylation of CE to form methoxyCE conjugates protects the CE from further oxidation to CE quinones. Based on the mean levels of 2-methoxyCE and 4-methoxyCE detected, this level of protection appeared to be greater in the controls (3.5 + 4.1 pmol/g tissue) than in the cases (1.9 + 3.2), although the differences were not statistically significant in this population (Figure 4). In contrast, highly significant differences can be observed in the formation of GSH conjugates by the CE quinones (Figure 4). As noted above, these conjugates are detected as Cys and NACys conjugates because the GSH is hydrolyzed in the tissue following the mercapturic acid biosynthesis pathway (22). The presence of these conjugates demonstrates that CE quinones have been formed in the tissue. At the time of this study, our HPLC analysis was not adequately developed to detect the depurinating CE–DNA adducts, although we have now worked out conditions to detect them. The finding of the CE–GSH conjugates, however, implies that the CE quinones could also have reacted with DNA present in the same cells to form adducts, and we used the CE–GSH conjugates detected in this study as surrogates for the formation of depurinating DNA adducts.

In the cases, both the level of CE–GSH conjugates (8.2 versus 2.6 pmol/g tissue, \( P = 0.003 \)) and the percentage of positive samples (57 versus 29%, \( P = 0.02 \)) were significantly higher than in the controls. This finding strongly supports the hypothesis that imbalances in estrogen metabolism lead to formation of E₁(E₂)-3,4-Q, which may react with DNA to form depurinating adducts. These DNA lesions generate apurinic sites, which could lead to mutations that may initiate cancer (7).

Similar profiles of estrogen metabolites and conjugates, as well as depurinating CE–DNA adducts, have been observed in target organs for estrogen-induced tumors in three animal models: male Syrian golden hamster kidney (20), male Noble rat prostate (26) and female ERKO/Wnt-1 mouse mammary gland (27). In male hamsters treated with E₂, the metabolic profile of estrogens was determined in the susceptible kidney target tissue and in the refractory liver (20). The kidney metabolic profile showed less methoxyCE than the liver and much more CE–GSH conjugates, suggesting that the estrogen metabolic profile in the kidney is more unbalanced than in the liver. Analysis of estrogen metabolites and conjugates in the non-susceptible ventral and anterior prostate, and the susceptible dorsolateral and perirethral prostate of rats treated with 4-OHE₂ or E₂-3,4-Q (26) revealed that the areas of the prostate susceptible to carcinoma induction have less protection by COMT, quinone reductase and GSH, thereby favoring reaction of E₁(E₂)-3,4-Q with DNA.

Female ERKO/Wnt-1 mice spontaneously develop mammary tumors despite the lack of functional estrogen receptor-α. Analysis of the estrogen metabolites and conjugates in the mammary tissue revealed significant imbalance in estrogen metabolism: 4-OHE₁(E₂) and 4-OHE₁(E₂)–GSH conjugates were detected, but not 2-OHE₁(E₂), 2-methoxyE₁(E₂) or 4-methoxyE₁(E₂) (27).

In the analysis of breast tissue from women with and without breast cancer reported here, we have also found greater imbalance in estrogen metabolism in tissue from women with breast carcinoma. This includes significantly higher levels of 4-OHE₁(E₂) and CE–GSH conjugates (Table I, Figures 3 and 4). These findings suggest the possibility of developing biomarkers of susceptibility to the initiation of breast cancer and strategies to prevent this disease.

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References


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