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Investigation of the cumulative body burden of estrogen-3,4-quinone in breast cancer patients and controls using albumin adducts as biomarkers

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HIGHLIGHTS

- We compared the body burden of estrogen quinones in Taiwanese women with breast cancer and controls.
- Serum E2-3,4-Q was inversely proportional to BMI among premenopausal women.
- Elevation of body burden of E2-3,4-Q may play a role in the development of breast cancer.

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ABSTRACT

Both 17β-estradiol-2,3-quinone (E_2-2,3-Q) and 17β-estradiol-3,4-quinone (E_2-3,4-Q) are reactive metabolites of estrogen. Elevation of E_2-3,4-Q to E_2-2,3-Q ratio is thought to be an important indicator of estrogen-induced carcinogenesis. Our current study compared the cumulative body burden of these estrogen quinones in serum samples taken from Taiwanese women with breast cancer ($n = 152$) vs healthy controls ($n = 75$) by using albumin (Alb) adducts as biomarkers. Results clearly demonstrated the presence of cysteinyl adducts of E_2-2,3-Q-4-S-Alb and E_2-3,4-Q-2-S-Alb in all study population at levels ranging from 61.7–1330 to 66.6–1590 pmol/g, respectively. Correlation coefficient between E_2-2,3-Q-4-S-Alb and E_2-3,4-Q-2-S-Alb was 0.610 for controls and 0.767 for breast cancer patients ($p < 0.001$). We also noticed that in premenopausal subjects with body mass index (BMI) less than 27, background levels of E_2-3,4-Q-2-S-Alb was inversely proportional to BMI with about 25% increase in E_2-3,4-Q-2-S-Alb per 5 kg/m^2 decrease in BMI ($p < 0.001$). In addition, we confirmed that mean levels of E_2-3,4-Q-2-S-Alb in breast cancer patients were ~5-fold greater than in those of controls ($p < 0.001$). Overall, this evidence suggests that disparity in estrogen disposition and the subsequent elevation of cumulative body burden of E_2-3,4-Q may play a role in the development of breast cancer.

1. Introduction

Considerable evidence indicates that genetic and environmental factors contribute to the risk of developing breast cancer. Mutations in the tumor suppressor genes BRCA1 and BRCA2 confer increased risks for breast cancer (Newman et al., 1988). Polymorphisms in estrogen disposition and DNA repair genes play roles in breast carcinogenesis and have been found to modify breast cancer risk (Okobia et al., 2009; Shin et al., 2007; Hung et al., 2005). Variation in estrogen bioactivation and deactivation genes can also cause imbalance in estrogen metabolism, resulting in accumulation of reactive quinone species and increased risk of developing breast cancer (Okobia et al., 2009; Shin et al., 2007). Additionally, increased serum estrogen and modulation of estrogen disposition are both associated with the development of breast cancer (Del Giudice et al., 1998; Yager and Davidson, 2006). Mitogenesis driven

Abbreviations: Alb, albumin; CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1; E_2, 17β-estradiol; E_2-2,3-Q, 17β-estradiol-2,3-quinone; E_2-3,4-Q, 17β-estradiol-3,4-quinone; E_2-2,3-Q-1-S-NAC and E_2-2,3-Q-4-S-NAC, reaction products of E_2-2,3-Q with *N*-acetyl-L-cysteine; E_2-3,4-Q-2-S-NAC, reaction products of E_2-3,4-Q with *N*-acetyl-L-cysteine; E_2-2,3-Q-1-S-Alb and E_2-2,3-Q-4-S-Alb, adducts resulting from reaction of E_2-2,3-Q with Alb; E_2-3,4-Q-2-S-Alb, adducts resulting from reaction of E_2-3,4-Q with Alb; E_2-2,3-Q-1-*S*-TFA and E_2-2,3-Q-4-*S*-TFA, trifluoroacetyl derivative of E_2-2,3-Q adduct after adduct cleavage; E_2-3,4-Q-2-*S*-TFA, trifluoroacetyl derivatives of E_2-3,4-Q adduct after adduct cleavage; EI, electron impact; GC–MS, gas chromatograph and mass spectrometer; [$^2H_5$]-E_2, E_2-2,4,16,16,17-d_5 ([$^2H_5$]-E_2); HPLC, high performance liquid chromatography; MSA, methanesulfonic acid; NAC, *N*-acetyl-L-cysteine; NCI, negative ion chemical ionization; SD, standard deviation; TFA, trifluoroacetyl; TFAA, trifluoroacetic acid anhydride.

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by estrogen receptor plays critical roles in estrogen carcinogenicity (Feigelson and Henderson, 1996). Conversion of 17β-estradiol (E_2) to the reactive metabolites, including 2-hydroxyestradiol (2-OH-E_2) and 4-hydroxyestradiol (4-OH-E_2), is mediated by CYP1A1 and CYP1B1 (Hayes et al., 1996; Martucci and Fishman, 1993; Spink et al., 1997). Both 2-OH-E_2 and 4-OH-E_2 may undergo oxidation process to generate quinones, including estrogen-2,3-quinone (E_2-2,3-Q) and estrogen-3,4-quinone (E_2-3,4-Q) (Butterworth et al., 1996; Cao et al., 1998). It is believed that accumulation of estrogen quinones, particularly E_2-3,4-Q, along with the subsequent generation of abasic sites as well as other pro-mutagenic DNA damage, contribute to the initiation of estrogen-induced carcinogenesis (Yager and Davidson, 2006; Bolton and Thatcher, 2008; Parl et al., 2009).

The effect of body mass index (BMI) on the risk of breast cancer depends on menopausal status. BMI has significant inverse associations with breast cancer among premenopausal women, but positive associations with breast cancer among postmenopausal women (van den Brandt et al., 2000; Michels et al., 2006; Weiderpass et al., 2004; Lahmann et al., 2003). The mechanisms underlying the relationship between BMI and breast cancer risk remain unclear, but high serum concentration of bioavailable estrogen may play roles in mediating estrogen homeostasis (Key et al., 2003). Some studies have revealed that sex hormone-binding globulin levels, aromatization of androstenedione in adipose tissue, and frequency of an ovulatory menstrual cycles may all contribute to the bio-availability of estrogen (Haffner et al., 1989; Kirschner et al., 1990; Key et al., 2003). This association between BMI and breast cancer risk may be modified by inherited differences in activation and deactivation of reactive intermediates of estrogen by various metabolizing enzymes. However, information regarding the joint effects of BMI and biomarkers of estrogen homeostasis on breast cancer risk has not been reported.

The aim of this study was to extent our previous investigation of estrogen quinone-derived adducts in serum Alb derived from breast cancer patients and controls to a larger scale. We also examined the relationships between burden of these estrogen quinones in human serum, BMI, and risk of breast cancer. In this work, we refined the original protocol to allow simultaneous analyses of estrogen quinone-derived adducts in serum Alb.

2. Materials and methods

2.1. Chemicals

TFAA, MSA, E_2, chloroform, *N*-acetyl-L-cysteine (NAC), L-ascorbic acid (99%), human serum Alb, and potassium nitrosodisulfonate were purchased from Sigma–Aldrich Inc. (St. Louis, MO 63178, USA). E_2-2,4,16,16,17-d_5 ([$^2H_5$]-E_2) was from C/D/N isotope (Canada H9R 1H1). Acetone, methyl alcohol, ethyl acetate, and acetonitrile were obtained from TEDIA (Charlotte, NC 288224, USA).

2.2. Synthesis of isotopically-labeled protein bound internal standards

Isotopically-labeled protein bound internal standards were synthesized according to the procedure previously described by Butterworth et al. (1996) with modifications (Chen et al., 2011). In brief, 5 mg (0.018 mmole) of [$^2H_5$]-E_2 (dissolved in 2 mL of acetone) were added to 3 mL of acetic acid. After the addition of 50 mg of potassium nitrosodisulfonate, the mixture was shaken for 15 min at room temperature. A second portion of potassium nitrosodisulfonate (50 mg) was added and the reaction was continued for another 15 min. Estrogen quinones were extracted from the solution three times with chloroform (2 mL × 3). Chloroform was removed under a gentle stream of N_2. Twenty microliter of acetonitrile was added to the residue and reactions were carried out by incubating estrogen quinone with 100 mg of human serum Alb at 37 °C for 2 h. The reactions were terminated by adding 10 mM of ascorbic acid (final concentration) and chilling in an ice bath. The modified proteins were purified by dialysis against 4 × 4 L of 1 mM ascorbic acid at 4 °C for 24 h using Spectra-Por 2 dialysis tubing (MWCO 12000–14000). The dialyzed proteins were lyophilized, weighed, and stored under −80 °C prior to use.

2.3. Synthesis of adducts of catechol estrogen with N-acetyl-L-cysteine

Authentic standards were synthesized following the procedure of (Cao et al., 1998). In brief, 6.9 mg of manganese dioxide (MnO_2) was added to 6.7 mg (0.023 mmole) of 2- and 4-hydroxy-17β-estradiol (dissolved in 5 mL of acetonitrile) (purchased from Sigma, St. Louis, MO 63178, USA) and the mixture was stored at −30 °C for 30 min. The reactions were initiated by incubating estrogen quinone with 7.6 mg (0.047 mmole) NAC at 37 °C for 60 min. The reactions were terminated by adding 10 mM of ascorbic acid (final concentration) and the final product was purified by reverse-phase HPLC as described by Chen et al. (2011).

2.4. Synthesis of adducts of estrogen quinones with human serum Alb

Estrogen quinone-bound Alb standards were synthesized according to the procedure previously described by Butterworth et al. (1996) with modifications (Chen et al., 2011). To 30 mg (0.11 mmole) of 17β-estradiol, 10 mL of acetone and then 16 mL of 10% acetic acid in water (v/v) were added. After the addition of 50 mg of potassium nitrosodisulfonate, the mixture was shaken for 15 min at room temperature. A second portion of potassium nitrosodisulfonate (50 mg) was added and the mixture was shaken for another 15 min. The estrogen quinones were extracted from the solution three times with chloroform (2mL × 3). Chloroform was removed under a gentle stream of N_2. 20 μL of acetonitrile was added to the residue and reactions were performed by adding estrogen quinones to a solution containing 100 mg human serum Alb for 30 min at 37 °C. The reactions were terminated by adding 10 mM of ascorbic acid (final concentration) and chilling in an ice bath. The modified protein was to serve as a positive control.

2.5. Subjects

The study population was recruited in a medical center located in central Taiwan. Women with breast cancer (n = 190) and female controls (n = 114) were recruited between May 2009 and November 2011. All of the participants provided sufficient venous blood for protein adduct analyses and completed questionnaires regarding age, occupation, disease history, cigarette smoking, and alcohol consumption, etc. Of those recruited, 152 breast cancer patients and 75 controls without any history of cancer were ultimately enrolled in this study where 84 breast cancer patients and 58 controls were premenopausal. None of the enrolled participants had history of alcohol-drinking, cigarette-smoking, or chemotherapy. Mean age was 40.0 for controls and 49.2 for breast cancer patients. The study protocol was reviewed by the Human Ethics Committee of the Changhua Christian Hospital, Taiwan. Each participant provided informed consent after receiving a detailed explanation of the study. All serum samples were maintained at −80 °C before protein isolation.

2.6. Isolation of human serum albumin

After bringing the serum to room temperature, Alb was isolated as follows. A saturated solution of $(NH_4)_2SO_4$ was added dropwise to the plasma until the final concentration of ammonium sulfate was 2.5 M (63% of saturation). The solution was mixed with a vortex mixer, and the immunoglobulins were removed by centrifuging for 30 min at 3000 *g*. The protein was purified by dialysis against 4 × 4 L of 1 mM ascorbic acid at 4 °C using Spectra-Por 2 dialysis tubing (MWCO 12,000–14,000). The dialyzed proteins were lyophilized, weighed, and stored at −80 °C prior to use.

2.7. Characterization and measurement of adducts

For estrogen quinones, the products of reactions between estrogen quinones and NAC are designated as E_2-2,3-Q-1-S-NAC, E_2-2,3-Q-4-S-NAC, and E_2-3,4-Q-2-S-NAC, respectively, and those with Alb as E_2-2,3-Q-1-S-Alb, E_2-2,3-Q-4-S-Alb, and E_2-3,4-Q-2-S-Alb, respectively. All cysteinyl adducts arising from estrogen quinones were assayed by the procedure as described by Chen et al. (2011). Briefly, after bringing samples to complete dryness in a vacuum oven (70 °C), we added 750 μL of TFAA and the reaction was allowed to proceed at 110 °C for 30 min. After cooling to room temperature, 20 μL of MSA was added and the mixture was heated at 110 °C for an additional 30 min. The unreacted TFAA was removed under a gentle stream of N_2. One and a half mL of hexane was added to the residue and the hexane layer was washed twice with 2 mL of 0.1 M Tris buffer (pH 7.4) and once with 1 mL of deionized water. After concentrating the samples to 50 μL, a 2-μl aliquot was analyzed by gas chromatograph and mass spectrometer (GC–MS). All analyses were conducted using a Agilent 6890 series GC coupled to a Agilent 5973N MS. A HP-5MS fused silica capillary column (30 m, 0.25-mm i.d., 0.25-μm film thickness) was used with He (99.999%) as the carrier gas at a flow rate of 1 ml/min. The MS transfer-line temperature was 250 °C and the chemical ionization reagent gas (methane) pressure was 2.3×10^{-4} Torr.

For characterization of adducts in the assay, our GC–MS was set to scan from m/Z 50 to m/Z 750 in electron impact (EI) and negative ion chemical ionization (NCI) modes. The ion source temperature was set at 150 °C and the injection-port temperature was 250 °C in all cases. The GC oven temperature was held at 75 °C for 2 min and increased at 6 °C/min to 145 °C, where it was held for 10 min. Late-eluting compounds were removed by increasing the oven temperature at 50 °C/min to 260 °C, where it was held for 5 min.