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Hemoglobin adducts as biomarkers of estrogen homeostasis: Elevation of estrogenquinones as a risk factor for developing breast cancer in Taiwanese Women

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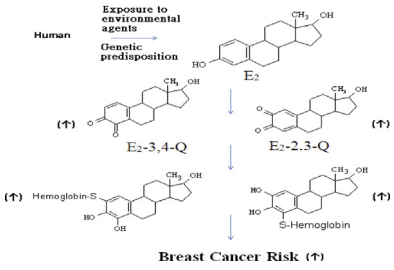
HIGHLIGHTS

- This study was to establish a method to analyze estrogen quinone-derived adducts.
- These adducts were measured in hemoglobin derived from breast cancer patients.
- A 6-fold increase in mean values of adducts in breast cancer patients was detected.
- Estrogen quinone hemoglobin adducts is an important indicator of breast cancer risk.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to establish a methodology to analyze estrogen quinone-derived adducts, including 17β-estradiol-2,3-quinone (E_2-2,3-Q) and 17β-estradiol-3,4-quinone (E_2-3,4-Q), in human hemoglobin (Hb). The methodology was then used to measure the levels of these adducts in Hb derived from female breast cancer patients ($n = 143$) as well as controls ($n = 147$) in Taiwan. Our result confirmed that both E_2-2,3-Q- and E_2-3,4-Q-derived adducts, including E_2-2,3-Q-4-S-Hb and E_2-3,4-Q-2-S-Hb, were detected in all breast cancer patients with median levels at 434 (215–1472) and 913 (559–2384) (pmol/g), respectively. Levels of E_2-2,3-Q-4-S-Hb correlated significantly with those of E_2-3,4-Q-2-S-Hb ($r = 0.622$–0.628, $p < 0.001$). By contrast, median levels of these same estrogen quinone-derived adducts in healthy controls were 71.8 (35.7–292) and 139 (69.1–453) (pmol/g). This translated to ~6-fold increase in mean values of E_2-2,3-Q-4-S-Hb and E_2-3,4-Q-2-S-Hb in breast cancer patients compared to those in the controls ($p < 0.001$). Our findings add further support to the theme that cumulative body burden of

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-Hb and E_2-2,3-Q-4-S-Hb, adducts resulting from reaction of E_2-2,3-Q with hemoglobin; E_2-3,4-Q-2-S-H, badducts resulting from reaction of E_2-3,4-Q with hemoglobin; 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-d5 ([$^2H_5$]-E_2); Hb, hemoglobin; 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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estrogen quinones is an important indicator of breast cancer risk. We hypothesize that combination of genetic events and environmental factors may modulate estrogen homeostasis and enhance the production of estrogen quinones which lead to subsequent generation of pro-mutagenic DNA lesions in breast cancer patients.

1. Introduction

Estrogen is a complete carcinogen. Evidence suggests that carcinogenicity of estrogen is mainly attributed to the receptor-driven mitogenesis mechanism. This includes bioactivation of estrogen and the subsequent formation of DNA damage (Bolton and Thatcher, 2008; Cavalieri and Rogan, 2011; Lavigne et al., 2001; Yager, 2000). CYP1A1 and CYP1B1 have been shown to mediate the conversion of 17β-estradiol (E_2) to reactive metabolites, including 2-hydroxyestradiol (2-OH-E_2) and 4-hydroxyestradiol (4-OH-E_2) (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 their respective 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). Both E_2-2,3-Q and E_2-3,4-Q can give rise to labile DNA adducts, leading to oncogenic mutations (Fig. 1) (Cavalieri et al., 1997, 2002; Convert et al., 2002; Lin et al., 2003; Zahid et al., 2006). It is believed that estrogen quinones play significant roles in the initiation of estrogen-induced tumorigenesis (Bolton and Thatcher, 2008; Cavalieri and Rogan, 2011; Parl et al., 2009; Yager and Davidson, 2006).

Evidence suggests that in Taiwan, the onset of breast cancer tends to occur at a younger age than in Western countries (Cheng et al., 2000). Development of biomarkers to recognize high-risk individuals is needed. Blood protein adducts, including albumin (Alb) and hemoglobin (Hb), seem to represent reliable biomarkers of exposure to environmental pollutants. Bio-monitoring blood protein adducts have been used as alternatives to DNA adducts for human biomarkers of several different classes of carcinogens (Skipper et al., 1994; Tornqvist et al., 2002; Waidyanatha and Rappaport, 2008). In our previous investigation, we analyzed estrogen quinone-derived protein adducts in human serum Alb derived from breast cancer patients in Taiwan (Chen et al., 2011; Lin et al., 2013). We used the Alb adducts of quinonoid metabolites of estrogen as biomarkers to assess the cumulative body burden of reactive estrogen quinones in these subjects. We concluded that cumulative body burden of E_2-3,4-Q is an important indicator of breast cancer risk. In this study, we gauge the body burden of estrogen quinones in human Hb by measuring the levels of estrogen quinone-derived Hb adducts. The advantages of extending the assay to Hb adducts is the long life span of red blood cells in human. We refined the original protocol to measure both E_2-2,3-Q and E_2-3,4-Q adducts in human Hb derived from Taiwanese breast cancer patients and healthy controls.

2. Materials and methods

2.1. Chemicals

Trifluoroacetic acid anhydride (TFAA), methanesulfonic acid (MSA), E_2, chloroform, *N*-acetyl-L-cysteine (NAC), L-ascorbic acid (99%), Hb, and potassium nitrosodisulfonate were purchased from Sigma–Aldrich Inc. (St. Louis, MO 63178, USA). E_2-2, 4, 16, 16, 17-d5 ([$^2H_5$]-E_2) was purchased 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 adducts of estrogen quinones with human globin

To 30 mg (0.11 mmol) 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 (3× 2 mL). 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 human globin (in 1× PBS; purchased from Sigma–Aldrich) for 30 min at 37 °C. The reactions were terminated by adding 10 mM of ascorbic acid (final concentration) and by chilling in an ice bath. The modified protein was to serve as a positive control.

2.3. Synthesis of isotopically labeled globin-bound internal standards

For the analysis of estrogen quinone-derived adducts, isotopically labeled globin-bound internal standards were synthesized and purified according to the procedure previously described by Chen et al. (2011).

2.4. Subjects

The study population was recruited in a suburban medical center in central Taiwan. Women with breast cancer (n = 190) and healthy female subjects (n = 205) were recruited between May 2009 and May 2012. All of the participants provided sufficient venous blood for protein adduct analyses and completed questionnaires regarding age, body mass index, occupation, disease history, cigarette smoking, alcohol consumption, and dietary habits. Of those recruited, 143 breast cancer patients and 147 controls (without any history of cancer) were ultimately enrolled in this study. None of the subjects had history of alcohol use, smoking, or chemotherapy. The mean ages were 41.7 ± 10.6 (median 41; range 23–69) for controls and 49.5 ± 7.4 (median 49; range 36–63) for cancer patients. Estrogen quinone-derived Hb adducts were analyzed in each group. The study protocol was reviewed by the Human Ethics Committee of the Changhua Christian Hospital, Taiwan (IRB-081219). Each participant provided informed consent after receiving a detailed explanation of the study. All red blood cell samples were maintained at −80 °C before protein isolation. Some of the hemoglobin samples obtained from breast cancer patients and controls were from the same patients involved in the previous study of the authors (Lin et al., 2013).

2.5. Isolation of globin

The red blood cell membranes were removed by centrifuging at 30,000 g at 5 °C for 20 min. The supernatant was purified by exhaustive dialysis (Spectra-Pore 1, 6000–8000 MWCO) against 4× 3.5 L of deionized water at 4 °C over 24 h (rather than by Sephadex chromatography). Globin was precipitated by adding the Hb solution dropwise to 10 volumes of ice-cold acetone containing 0.1% HCl, washed twice with ice-cold acetone, lyophilized, and stored under −80 °C.

2.6. Characterization and measurement of adducts

All cysteinyl adducts arising from estrogen quinones were assayed by the procedure described by Chen et al. (2011). Briefly, to an 8-mL vial containing 10 mg of protein, isotopically labeled protein-bound internal standards for estrogen quinones were added. After bringing samples to complete dryness in a vacuum oven (70 °C), per-acetylation was achieved with the addition of 750 μL of TFAA and the reaction was allowed to proceed at 110 °C for 30 min. After cooling to room temperature, adducts were cleaved by adding 20 μL of MSA and the mixture was heated at 110 °C for an additional 30 min. The un-reacted anhydride 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 an Agilent 6890 series GC coupled to an 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.