

DNA Adduct Formation of 17 $\beta$ -Estradiol in MCF-7 Human Breast Cancer CellsSang Yoon Lee,<sup>†</sup> Ji-Su Kim, and Sang Soo Hah\*

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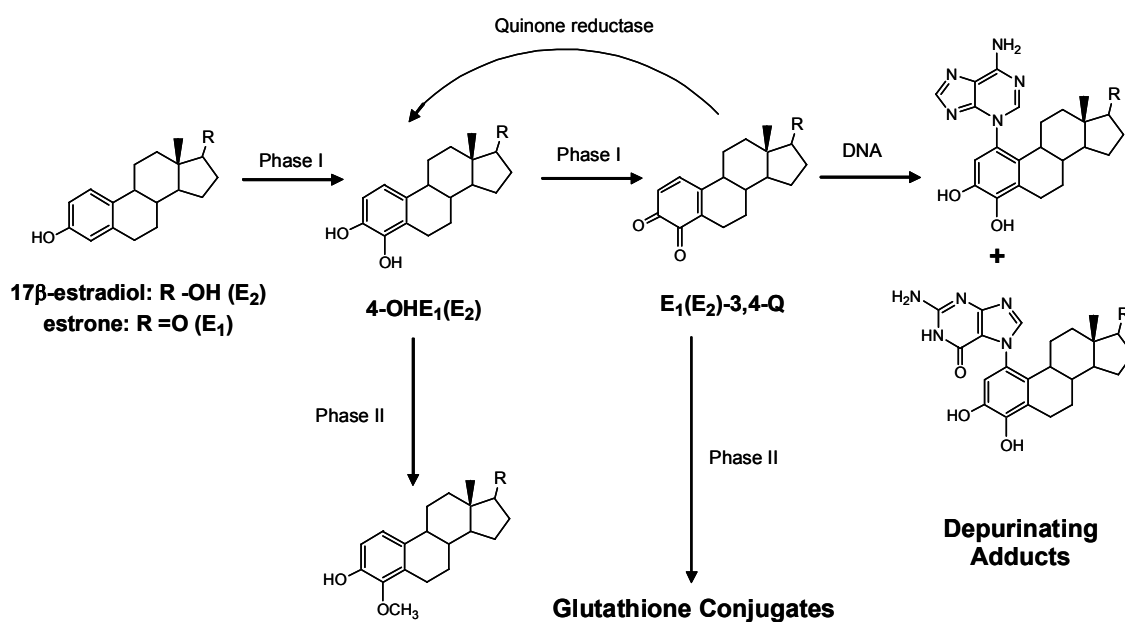
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While estrogens are endogenous hormones that are involved in the development and maintenance of reproductive organs, tissue differentiation, and gene expression,<sup>1</sup> prolonged unopposed exposure of cells to 17 $\beta$ -estradiol (E<sub>2</sub>) or estrone (E<sub>1</sub>), for example, is known to cause the production of reactive oxygen species (ROS), such as superoxide and hydroxyl radical, thus leading to exogenous induction of oxidative stress, and estrogen exposure is now a widely accepted risk factor in breast cancer development.<sup>2,3</sup> The mechanisms through which estrogens induce breast carcinogenesis have not been definitely unraveled, although corresponding catechol and quinone metabolite formation is known to produce ROS. There are two main hypotheses about how estrogen initiates cancer: one through estrogen receptor-mediated processes and the other by DNA damage caused from the interaction of specific estrogen metabolites with DNA (see Figure 1 for the major pathway of estrogen activation in the formation of depurinating estrogen-DNA adducts).<sup>4</sup> Though the metabolism of estrogen is extremely complex involving many

enzymes, which produce a multitude of metabolites, it is generally accepted that genotoxicity may be caused by cytochrome P450-mediated oxidation of catechol estrogens to quinone formation that react with DNA to form depurinating estrogen-DNA adducts. The estradiol-3,4-quinone (E<sub>2</sub>-3,4-Q) is also reported to react directly with DNA *in vitro* to form covalent estrogen-DNA adducts.<sup>5</sup> One of these DNA lesions is the 4-hydroxyestradiol-*N*<sup>7</sup>-guanine adducts, which undergoes depurination at the glycosidic bond creating potentially mutagenic abasic sites.<sup>6</sup>

Limited sensitivity of existing assays, however, has prevented investigation of whether estrogen can covalently form DNA adducts in cells or involved in the genotoxic potential of estrogens. This work sought to verify covalent estrogen-DNA adducts at nanomolar doses using accelerator mass spectrometry (AMS), a highly sensitive technique with origins in geochemistry for radiocarbon dating, since AMS affords sub-picomole to zeptomole <sup>14</sup>C per mg total carbon sensitivity with a few percent precision, depending on experimental conditions.<sup>7,8</sup> AMS is



**Figure 1.** Major pathway of estrogen activation in the formation of depurinating estrogen-DNA adducts. Cytochrome P450 and peroxidases are involved in Phase I to form catechol estrogen 4-hydroxyestradiol (4-OHE<sub>2</sub>) or 4-hydroxyestrone (4-OHE<sub>1</sub>), followed by formation of estradiol-3,4-quinone (E<sub>2</sub>-3,4-Q) or estrone-3,4-quinone (E<sub>1</sub>-3,4-Q) which can be converted back to 4-OHE<sub>1</sub>(E<sub>2</sub>) by quinone reductase. Catechol-*o*-methyltransferase (COMT) and glutathione (GSH) participate in Phase II to form *O*-methylated estrogen derivatives and glutathione conjugates, respectively.

specific only to the labeled compound in any chemical or biological medium. In this study, we report the use of AMS to quantify covalent E<sub>2</sub>-DNA adducts in estrogen receptor-positive MCF-7 human breast cancer cells in the absence and presence of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG), one of the most prevalent lesions found in DNA,<sup>9</sup> and we provide evidence of E<sub>2</sub>-DNA adducts at physiologically-relevant E<sub>2</sub> concentrations, which represents a novel pathway for E<sub>2</sub> metabolism in MCF-7 cells.

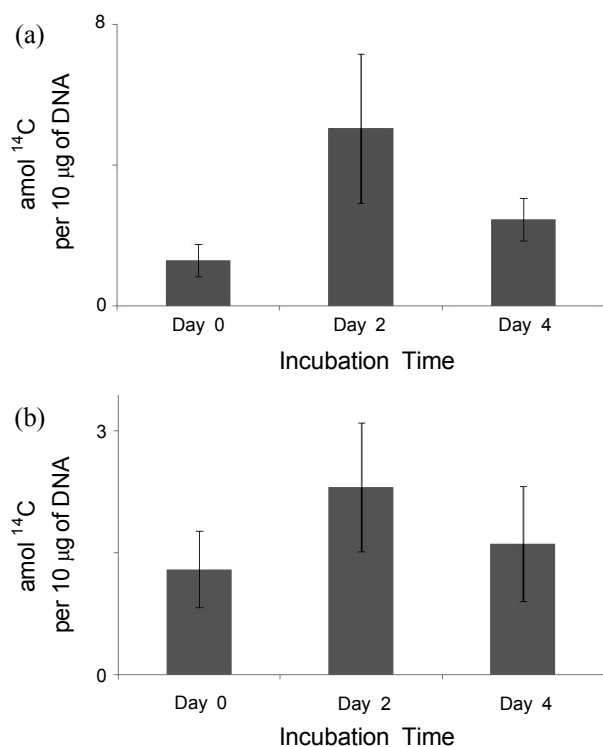
To quantify estrogen-DNA adducts in MCF-7 cells at nanomolar concentrations, we used <sup>14</sup>C-labeled E<sub>2</sub> ([<sup>14</sup>C]E<sub>2</sub>) and dividing cells were dosed with [<sup>14</sup>C]E<sub>2</sub> 2 days before confluence, and total genomic DNA was isolated by using conditions according to the literature.<sup>9</sup> Figure 2(a) shows that adduct formation of radiolabeled E<sub>2</sub> with DNA of MCF-7 cells reached a maximum of ~5 amol/10 µg of DNA after 2 days of incubation and decreased ~2-fold by day 4, presumably due to DNA depurination and/or DNA repair competing with radiolabeled E<sub>2</sub> adduct formation with DNA. The maximum adduct formation with DNA of MCF-7 cells corresponds to ~400 molecules per cell or ~0.6 molecules per 10<sup>7</sup> normal nucleotides. Clearly, this experiment demonstrates that E<sub>2</sub> can be converted to active cellular metabolites to form DNA adducts at physiologically-relevant conditions. This experiment, to our best knowledge, represents the first report of direct measurement of E<sub>2</sub> adduct formation with DNA *in vivo*. We are now investigating enzymatic digestion of the radiolabeled DNA to 2'-deoxynucleosides to identify estrogen-DNA adducts under a variety of conditions.

The MCF-7 dosing experiments were repeated in the presence

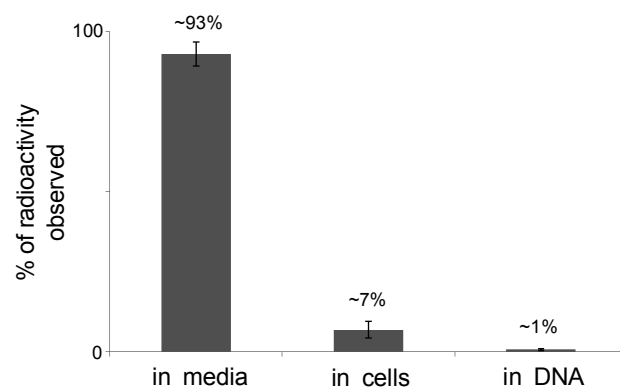
of 10.8 pmol 8-oxodG, and <sup>14</sup>C content of extracted DNA was measured by AMS (Figure 2(b)). The conditions of this experiment were closely similar with our previous work, which described that E<sub>2</sub> could induce oxidative stress up-regulated the nucleotide pool cleansing enzyme MTH1 and possibly other Nudix-related pyrophosphohydrolases. In our previous study, we used [<sup>14</sup>C]8-oxodG in the presence of 100 nM E<sub>2</sub> to understand the impact of E<sub>2</sub> on the rate of 8-oxodG incorporation into DNA of MCF-7 cells, demonstrating that [<sup>14</sup>C]8-oxodG incorporation into DNA is slowed in an E<sub>2</sub>-dependent manner, although these estrogen receptor-positive cells proliferate in response either to 8-oxodG or E<sub>2</sub> exposure at physiologically-relevant concentrations (data not shown). Interestingly, the degree of E<sub>2</sub>-DNA adduct formation was affected by exogenous 8-oxodG and decreased ~2-fold, as shown in Figure 2(b). We will investigate the up-regulated mechanism of E<sub>2</sub> induced by 8-oxodG in the near future.

To better understand the uptake and fate of the [<sup>14</sup>C]E<sub>2</sub> in the cells, we measured the distribution of the compound in various components of the cell culture system including the medium, cells, and purified DNA (Figure 3). Cells were incubated in the presence of 300 dpm of [<sup>14</sup>C]E<sub>2</sub> (0.5 nmol total dose of E<sub>2</sub> in 5 mL of media) for 2 days. AMS samples were prepared in triplicate from 20 µL aliquots each of the medium, lysed cells, and purified DNA. The AMS data allowed calculation of the fractional amount of <sup>14</sup>C in each sample. Of the total radiocarbon, 93.2 ± 3.7% persisted in the medium. The remaining radiocarbon was localized on or inside the cells, with 0.75 ± 0.32% uptake of <sup>14</sup>C onto nuclear DNA. This amount of radiocarbon in the DNA would be difficult to be characterized by standard scintillation counting (a few disintegration per minute) or other types of mass spectrometry, but it was easily quantifiable by AMS. The mass distribution data indicate that a substantial amount of the exogenous [<sup>14</sup>C]E<sub>2</sub> was taken into the cells and adducted onto the DNA.

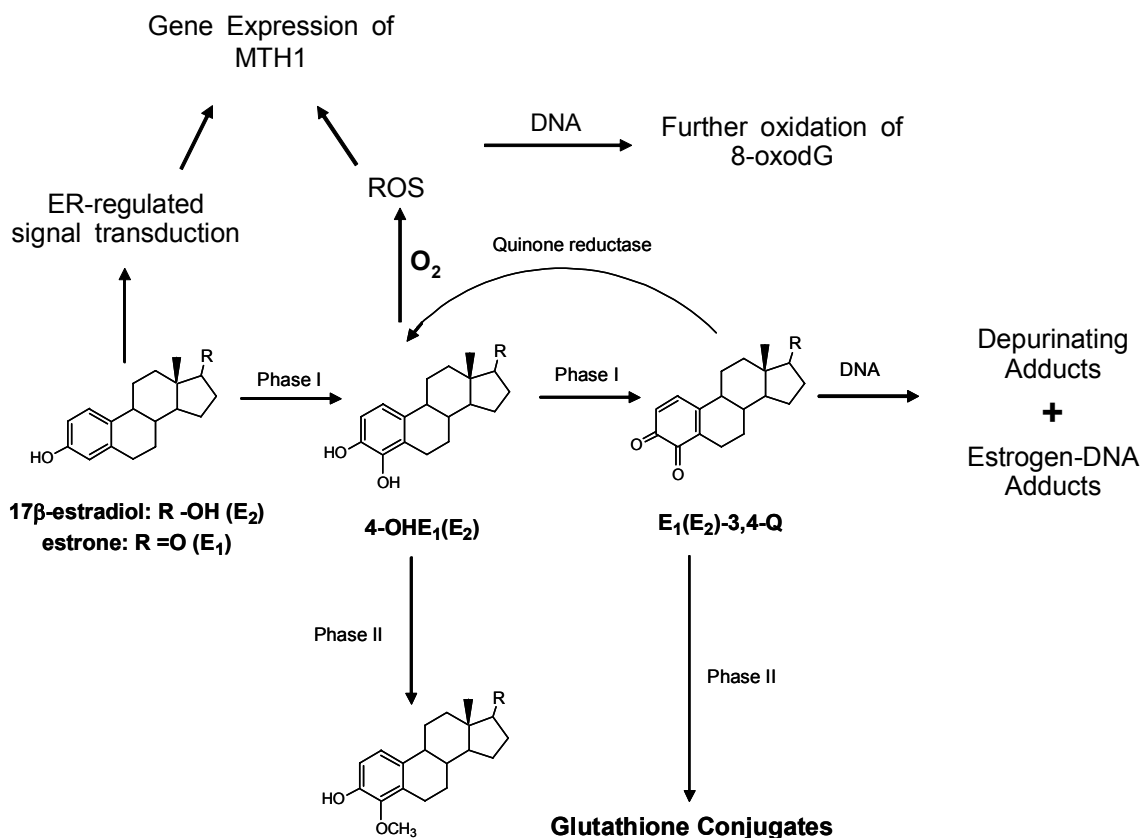
Although the exact mechanism by which estrogens exert their genotoxic activity is still uncertain, the dominant mechanism of action appears to involve direct reaction of active estrogen metabolites with DNA, consistent with observed DNA depurination at the glycosidic bond. Based on the data reported here, however, it is likely that E<sub>2</sub> may directly react with DNA in



**Figure 2.** Measurement of E<sub>2</sub> adducts in DNA of growing MCF-7 human breast cancer cells in the absence (a) and presence (b) of 8-oxodG for 0, 2, and 4 days, respectively. The ratio of <sup>14</sup>C to total carbon contained in the purified genomic DNA was measured by accelerator mass spectrometry (AMS).



**Figure 3.** Mass distribution in growth media, in MCF-7 cells, and in extracted DNA, respectively, after a 2-day incubation of MCF-7 cells with 300 dpm of [<sup>14</sup>C]E<sub>2</sub>.



**Figure 4.** Proposed mechanism of E<sub>2</sub> metabolism in MCF-7 cells. In addition to Figure 1 caption, E<sub>2</sub> may participate in estrogen receptor-regulated signal transduction as well as cause the production of reactive oxygen species (ROS), leading to exogenous induction of oxidative stress, followed by activation of gene expression of MTH1, a pyrophosphohydrolase able to hydrolyze 8-oxodG triphosphate to 8-oxodG monophosphate in the nucleotide pool. E<sub>2</sub> may also directly react with DNA in cells to form covalent estrogen-DNA adducts.

cells to form covalent estrogen-DNA adducts (Figure 4), suggesting that E<sub>2</sub> acts by many different mechanisms to cause genotoxicity, and this would be consistent with its broad spectrum activity.

In conclusion, although our data account for only a small percentage of total E<sub>2</sub>, they provide direct evidence of E<sub>2</sub>-DNA adducts at physiologically-relevant E<sub>2</sub> concentrations, which represents a novel pathway for E<sub>2</sub> metabolism in cells and thus indicates a potent mechanism of action that may be responsible for genotoxicity of estrogens. Such an experiment has not been previously possible due to lack of sufficient analytical sensitivity.

### Experimental Section

**General Methods.** Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. Radiocarbon-labeled [<sup>14</sup>C]E<sub>2</sub> was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), of which the specific activity was 55 mCi/mmol. All experiments were performed in triplicate.

**Cell Culture and Dosing MCF-7 Human Breast Cancer Cells with E<sub>2</sub> Followed by DNA Extraction.** MCF-7 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in tissue culture plates

at 37 °C in an atmosphere of 5% CO<sub>2</sub> in DMEM-F-12 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Exponentially growing cells were cultured following standard procedures until approximately 50% confluence was reached. Cells (50,000) were plated on day 0 and harvested by means of trypsinization (0.5 mL of 0.25% trypsin solution for 5 min at 37 °C), and the resulting single cell suspension was plated into tissue culture plates. Cells were counted by using a Coulter Counter. Each plate was dosed, in the absence and presence of 8-oxodG (10.8 pmol/5 mL or 2.16 nM), with E<sub>2</sub> (100 nM), which contained 300 dpm (2.16 pmol) [<sup>14</sup>C]E<sub>2</sub> in 5 mL of media ([<sup>14</sup>C]E<sub>2</sub> comprises 0.00043% of the total E<sub>2</sub> dosed) with 1% DMSO. Triplicate plates were incubated with the labeled estrogen for 0, 2, and 4 days. At sampling time points, the media was removed and the cells were washed three times with phosphate-buffered saline (PBS), followed by trypsinization. The isolated cells were either directly assayed for radiocarbon content or subjected to lysis and DNA extraction.<sup>9</sup>

**AMS Measurement.** The radiocarbon content in cells and extracted DNA were measured by AMS according to the literature.<sup>9</sup> Briefly, AMS samples were prepared in triplicate from purified DNA (10 µg) after addition of carrier carbon in the form of 1 µL of tributyrin, and the ratio of <sup>14</sup>C to <sup>12</sup>C in a biological sample that has been converted to solid carbon (graphite

or fullerene) for analysis using a two-step oxidation-reduction process was measured.<sup>10</sup> Graphite targets were measured at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory. Calculation of the number of attomole (amol) of <sup>14</sup>C per 10 µg of extracted cellular DNA was performed using the conversion factor of 1 Modern equals 97.8 amol of <sup>14</sup>C per mg of total carbon.

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