Metabolism and DNA binding studies of 4-hydroxyestradiol and estradiol-3,4-quinone in vitro and in female ACI rat mammary gland in vivo

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Studies of estrogen metabolism, formation of DNA adducts, carcinogenicity, cell transformation and mutagenicity have led to the hypothesis that reaction of certain estrogen metabolites, predominantly catechol estrogen-3,4-quinones, with DNA can generate the critical mutations initiating breast, prostate and other cancers. The endogenous estrogens estrone (E1) and estradiol (E2) are oxidized to catechol estrogens (CE), 2- and 4-hydroxylated estrogens. In general, the major metabolites are 2-CE, whereas the 4-CE are the minor ones. The pathway to give 4-CE is the one that leads to the endogenous carcinogenic catechol estrogen-3,4-quinones (Figure 1). 4-Hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) are formed from estrone (E1) and estradiol (E2), which are biochemically interconvertible by the enzyme 17β-estradiol dehydrogenase (Figure 1). The most common pathway of conjugation of 4-OHE1(E2) in extracellular tissues occurs by O-methylation, which is catalyzed by the ubiquitous catechol-O-methyltransferase (COMT) (24). This inactivating pathway is in competition with the activation of CE to semiquinones (not shown in Figure 1) and quinones (Figure 1): these oxidations are catalyzed by peroxidases and cytochrome P450. The quinones can be inactivated by formation of glutathione (GSH) conjugates and/or by reduction to CE by quinone reductase. If, however, these two processes are insufficient, the CE-3,4-quinones can react with DNA to form primarily depurinating adducts. These adducts can generate the critical mutations that initiate cancer (Chakravarti et al., Oncogene, 2001, 20, 7945; Chakravarti et al., Proc. Am. Assoc. Cancer Res., 2003, 44, 180).

Introduction

Experiments on estrogen metabolism (1–7), formation of DNA adducts (8–12), carcinogenicity (13–16), cell transformation (17–19) and mutagenicity (20–22) have led to the hypothesis that certain estrogen metabolites, predominantly catechol estrogen-3,4-quinones, react with DNA and can generate the critical mutations initiating breast, prostate and other cancers (12).

Among the major metabolites of the estrogens are the catechol estrogens (CE), 2- and 4-hydroxylated estrogens. In general, the major metabolites are the 2-CE, whereas the 4-CE are the minor ones. The pathway to give 4-CE is the one that leads to the endogenous carcinogenic catechol estrogen-3,4-quinones (Figure 1). 4-Hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) are formed from estrone (E1) and estradiol (E2), which are biochemically interconvertible by the enzyme 17β-estradiol dehydrogenase (Figure 1). The most common pathway of conjugation of 4-OHE1(E2) in extracellular tissues occurs by O-methylation, which is catalyzed by the ubiquitous catechol-O-methyltransferase (COMT) (24). This inactivating pathway is in competition with the activation of CE to semiquinones (not shown in Figure 1) and quinones (Figure 1): these oxidations are catalyzed by peroxidases and cytochrome P450. The quinones can be inactivated by formation of glutathione (GSH) conjugates and/or by reduction to CE by quinone reductase. If, however, these two processes are insufficient, the CE-3,4-quinones can react with DNA to form primarily depurinating adducts. These adducts can generate the critical mutations that initiate cancer (Chakravarti et al., Oncogene, 2001, 20, 7945; Chakravarti et al., Proc. Am. Assoc. Cancer Res., 2003, 44, 180).

To determine the possible DNA adducts of E1(E2)-3,4-Q, standard adducts were synthesized by reaction of these quinones with dG, dA and the nucleobase Ade (9,25). Reaction of E1(E2)-3,4-Q with dG produces the depurinating adduct 4-hydroxyE1(E2)-1-N7Gua ([4-OHE1(E2)-1-N7Gua] by 1,4-Michael addition (Stack et al., Chem. Res. Toxicol., 1996, 9, 851). We report here that reaction of E1(E2)-3,4-Q with Ade results in the formation of 4-OHE1(E2)-1-N3Ade by 1,4-Michael addition. The N7Gua and N3Ade depurinating adducts formed both in vitro and in rat mammary gland in vivo were analyzed by HPLC with electrochemical detection and, for some samples, by LC/MS/MS. When E2-3,4-Q was reacted with DNA in vitro, the depurinating adducts 4-OHE2(E2)-1-N3Ade and 4-OHE2(E2)-1-N7Gua, which are rapidly lost from DNA by cleavage of the glycosyl bond, were formed (>99% of the total adducts), as well as traces of stable adducts, which remain in DNA unless removed by repair. Similar results were obtained when 4-OHE2 was oxidized by horseradish peroxidase, lactoperoxidase, tyrosinase or phenobarbital-induced rat liver microsomes in the presence of DNA. When 4-OHE2 or E2-3,4-Q was injected into the mammary glands of female ACI rats in vivo and the mammary tissue was excised 1 h later, the depurinating adducts 4-OHE2(E2)-1-N3Ade and 4-OHE2-1-N7Gua constituted >99% of the total adducts formed. In addition, 4-OHE2 conjugates formed by reaction of E2-3,4-Q with glutathione were also detected. These results demonstrate that the 4-CE are metabolized to CE-3,4-Q, which react with DNA to form primarily depurinating adducts. These adducts can generate the critical mutations that initiate cancer (Chakravarti et al., Oncogene, 2001, 20, 7945; Chakravarti et al., Proc. Am. Assoc. Cancer Res., 2003, 44, 180).
This article reports the synthesis and structure determination of 4-OHE(E2)-1-N3Ade adducts, and the formation of 4-OHE(E2)-1-N7Gua adducts in DNA in vitro and in the mammary gland of female ACI rats in vivo.

Materials and methods

Materials
2-OHE1, 2-OHE2, 4-OHE1, 4-OHE2, E1-3,4-Q and E2-3,4-Q were prepared as described previously (32–34). The 4-OHE(E2)-2-glutathione (SG), 4-OHE(E2)-2-cysteine (Cys) and 4-OHE(E2)-2-N-acetylcysteine (NACys) were synthesized according to the procedure of Cao et al. (35). 4-OHE(E2)-1-N7Gua was synthesized according to Stack et al. (25). 16α-OHE1 and methoxy derivatives of CE were purchased from Steraloids (Newport, RI). Ade was purchased from Aldrich (Milwaukee, WI). Estriol (16α-OHE2), ascorbic acid, horseradish peroxidase (Type VI), lactoperoxidase, tyrosinase, H2O2 and NADPH were purchased from Sigma Chemicals (St Louis, MO). Liver microsomes from phenobarbital-induced female Sprague–Dawley rats (Harlan, Indianapolis, IN) were prepared by a previously published method (36). Female ACI rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN). Bond Elut Certify II SPE cartridges (200 mg) were purchased from Varian (Palo Alto, CA).
Synthesis of 4-OHE1-1-N3Ade and 4-OHE2-1-N3Ade

To a suspension of activated MnO2 (119 mg, 1.37 mmol) in 5 ml of dimethylformamide (DMF) at 0°C was added 4-OHE2 (60 mg, 0.21 mmol). After 10 min of stirring at 0°C, the resulting E2-3,4-Q in DMF was filtered, directly added dropwise into a stirred solution of Ade (170 mg, 1.26 mmol) dissolved in 5 ml of acetic acid and water (1:1, v/v), and allowed to react for ~5 h with stirring at room temperature. The solution was then filtered and analyzed by HPLC.

Analytical HPLC was conducted on a Waters (Milford, MA) 2690 Separations Module equipped with a Waters 990 photodiode array detector; the eluent was monitored at 270 nm. A YMC ODS-AQ 5µm 120-A column (20 x 250 mm), Morris Phenom, NJ, was used with 70% methanol for 10 min, then a 35-min linear gradient to 65% methanol, followed by a 15-min linear gradient to 100% methanol at a flow rate of 1 ml/min. To purify the synthesized adduct, preparative HPLC was conducted using a YMC ODS-AQ 5µm 120-A column (20 x 250 mm) eluted with 50% methanol in water (containing 0.4% acetic acid) for 45 min, followed by a 5-min linear gradient to 75% methanol and a 10-min linear gradient to 100% methanol at a flow rate of 8 ml/min. The yield of 4-OHE1-1-N3Ade was 57%. 4-OHE1-1-N3Ade was synthesized by following the same procedure, and the yield was 44%.

4-OHE2-1-N3Ade

4-H NMR (600 MHz, DMSO-d6, TMS): 8.64 (s, 1H, 2-H of catechol, ß-isomer), 6.17 (s, 1H, 2-H of catechol, ß-isomer), 6.30 (bs, 2H, NH2 of Ade, exchangeable with D2O, ß-isomer), 6.75 (bs, 2H, NH2 of Ade, exchangeable with D2O, ß-isomer), 7.72 (bs, 2H, 2-H of Ade, ß-isomer), 7.74 (bs, 1H, 2-H of Ade, ß-isomer), 7.86 (bs, 2H, 3-OH, 4-OH of catechol, exchangeable with D2O), 8.13 (s, 1H, 8-H of Ade, ß-isomer), 8.24 (s, 1H, 8-H of Ade, ß-isomer). High resolution MS (HRMS), [M + H]+, C22H25N5O3: calcd m/z 422.2192, obsd m/z 422.2219, a difference of 0.7 ppm.

4-OHE1-1-N3Ade

4-H NMR (600 MHz, DMSO-d6, TMS): 8.64 (s, 1H, 2-H of catechol, ß-isomer), 6.67 (s, 1H, 2-H of catechol, ß-isomer), 7.72 (s, 1H, 2-H of Ade, ß-isomer), 7.74 (s, 1H, 2-H of Ade, ß-isomer), 8.00 (bs, 4H, 3-OH, 4-OH of catechol, NH2 of Ade, exchangeable with D2O), 8.23 (s, 1H, 8-H of Ade, ß-isomer), 8.34 (s, 1H, 8-H of Ade, ß-isomer). HRMS, [M + H]+, C22H25N5O3: calcd m/z 420.2036, obsd m/z 420.2039, a difference of 0.7 ppm.

Cova lent binding of E2-3,4-Q and enzymatically activated 4-OHE1 to DNA in vitro

E2-3,4-Q or enzymatically activated 4-OHE1 was bound to DNA according to previously published procedures (8). E2-3,4-Q (3.5 µmol/50 µl DMSO) was incubated with 10 ml of 3 mM calf thymus DNA in 0.067 M sodium-potassium phosphate (pH 7.0) for 2 h at 37°C. 4-OHE1 was bound to DNA in 15-ml reaction mixtures catalyzed by horseradish peroxidase, lactoperoxidase, tyrosinase or phenobarbital-induced rat liver microsomes during 2 h of incubation at 37°C. For peroxidase-catalyzed reactions, the mixtures contained 3 mM calf thymus DNA in 0.067 M sodium-potassium phosphate (pH 7.0), 0.5 mM H2O2, 4-OHE1 (3.5 µmol/50 µl DMSO) and 100 µg/ml horseradish peroxidase (31 U, type VI) or 100 µg/ml lactoperoxidase (9 U). The tyrosinase reaction mixtures contained 3 mM DNA in 0.067 M sodium-potassium phosphate (pH 7.0), 100 µg/ml tyrosinase (480 U, from mushrooms) and were incubated in uncapped tubes. For the microsomal-catalyzed reactions, 15-ml mixtures contained 3 mM DNA in 150 mM Tris–HCl (pH 7.5), 150 mM KCl, 5.5 mM MgCl2, 4-OHE1 (3.5 µmol/50 µl DMSO), 1 mg/ml micromolar protein and 1 mM 2-mercaptoethanol. Determination of hydroperoxide formation in the 4-OHE1 reaction mixtures was accomplished with either no enzyme or no cofactor. At the end of the incubation, a 1-ml aliquot was removed for analysis of stable adducts after purification of DNA. Two volumes of ethanol was added to the remainder of the mixture to precipitate DNA, and the supernatant was used to analyze depurinating adducts.

Analysis of depurinating adducts from in vitro incubations by HPLC

Supernatants from the in vitro reactions were evaporated to dryness under vacuum. The residues were dissolved in 1 ml of DMSO/methanol (1:1) and analyzed first by preparative HPLC on a Waters 2690 Separations Module equipped with a YMC ODS-AQ 5µm 120-A column (10 x 250 mm), which was eluted with 30% methanol in water for 20 min, followed by a 20-min linear gradient to 52% methanol in water, 10 min at 52% methanol and then 5 and 10-min linear gradient to 100% methanol, which was continued for 10 min at a flow rate of 4 ml/min. An initial run was conducted with the standard adducts to establish the retention times of 4-OHE1-1-N3Ade (~36 min) and 4-OHE2-1-N7Gua (~41 min). Then the DMSO/methanol extracts were separated and fractions were collected at the retention times of the standard adducts. The solvent in the collected fractions was removed under vacuum, and the residues were dissolved in 0.5 ml of methanol. A 50-µl aliquot of the fraction was then analyzed by HPLC on an ESA CoulChem electrochemical detector (Chelmsford, MA) equipped with a YMC ODS-AQ 5µm, 120-A column (4.6 x 250 mm), which was eluted with 30% acetonitrile in 50 mM ammonium phosphate, pH 3.0, at a flow rate of 1 ml/min. The remainder of the fraction was analyzed by liquid chromatography/MS (LC/MS).

Analysis of stable adducts by 32P-postlabeling

32P-postlabeling analysis of stable DNA adducts was carried out with 8 µg DNA (40 x 13 cm polyethyleneimine-cellulose plates. The experimental procedures for the enzymatic hydrolysis of DNA (except that the T4 kinase reaction was carried out at pH 8.2), and the D1, D2 and D4 chromatographic steps were as described previously (32), but the D3 direction of chromatographic separation was modified. After the D2 chromatographic step, the adducts were located by autoradiography and the chromatographic plate was cut into two equal halves. A paper wick was attached to the lower half, which was developed overnight (~15 h) with 0.7 M sodium acetate and 7 M urea, pH 6.4. The upper half of the plate was developed with 0.35 M sodium phosphate, 3.5 M urea, pH 6.4. After D4 development, the adduct spots on both halves were visualized by autoradiography and quantified by liquid scintillation counting of 20 spots.

Treatment of rats

Groups of five female ACI rats (10 weeks old) were lightly anesthetized with ether, and the mammary region was shaved. They were then treated with either 4-OHE1 or E2-3,4-Q by intramammary injection (with a 27-gauge needle) under the skin of the region of the fourth and fifth mammary glands on both the right and left sides, at a dose of 200 nmol/gland in 20 µl of DMSO. Animals used for control were treated with 20 µl of DMSO. After 1 h, the animals were killed and the mammary gland areas were excised. Mammary tissue was minced, ground in liquid nitrogen and split into two samples weighing ~1.8 g each. The smaller sample was used for isolation of DNA and analysis of stable adducts by the 32P-postlabeling method, whereas the larger sample was used for analysis of metabolites and conjugates.

Extraction of estrogen derivatives

Ground mammary tissue was divided into three portions of ~2.5 g each, and each was extracted with 50 ml of hexane to remove lipids. After centrifugation and evaporation of hexane, the pellet was suspended in 3 ml of 50 mM ammonium acetate, pH 5.0. One-third of the tissue was analyzed in each run. Methanol was added to all fractions such that the final concentration of methanol was 60%. The supernatant was diluted to an approximate final concentration of 25% methanol with 30 mM ammonium acetate buffer, pH 4.4, containing 2 mg/ml ascorbic acid, and the resulting solution was applied to a Certify II Sep-Pak cartridge. The cartridge was first eluted with 3 ml of the buffer, followed by elution with 2 ml each of 20% and 40% and 3 ml of 70% methanol in buffer. The 70% fraction was analyzed by HPLC equipped with an ESA CoulArray electrochemical detector and by LC/MS.
evaporated and the residues were dissolved in DMSO/methanol (1:1). The depurinating DNA adducts in the extracts were initially purified by preparative HPLC by using a methanol/0.4% acetic acid gradient. The gradient started with 30% methanol for 1 min at a flow rate of 1 ml/min, followed by a flow rate of 4 ml/min for 10 min, then a 35-min linear gradient to 65% methanol, and a 5-minute linear gradient to 100% methanol. Collected fractions of adducts were analyzed by HPLC equipped with an ESA CoulChem II electrochemical detector, as well as by MS.

**HPLC analysis of CE derivatives in mammary tissue**

HPLC analysis was carried out on a reverse-phase Luna (2) C18 column (Phenomenex, Torrance, CA, 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 multi-channel CoulArray detector.

Based on a previously described procedure (11), the compounds were separated using a 50-min linear gradient starting with 100% acetonitrile/methanol/0.1 M ammonium acetate, pH 4.4, 15:5:80% acetonitrile/methanol/0.1 M ammonium acetate, pH 4.4, 50:20:30 at a flow rate of 1 ml/min. The serial array of 12 coulometric electrodes was set at potentials between 0 and 590 mV (0, 40, 80, 130, 180, 230, 280, 340, 400, 470, 530 and 590 mV, respectively). The system was controlled and data acquired and processed using CoulArray software. Peaks were identified by both retention times and by peak height ratios between the dominant peak and the peaks in the two adjacent channels. The analytes were quantified by comparison of peak heights with known amounts of standards.

**LC-MS/MS analysis of estrogen metabolites, conjugates and depurinating adducts**

Fractions from preparative HPLC were analyzed by LC-MS to confirm the identification of the depurinating DNA adducts, whereas fractions obtained by solid phase extraction were analyzed by LC-MS in an effort to confirm the results of the coulometric detection of the metabolites and theother conjugates. For the *in vitro* experiments, all LC-MS and LC-MS/MS data were collected using a PE Sciex API 365 (PE Sciex, Foster City, CA) triple quadrupole mass spectrometer equipped with a Sciex turboion spray inlet. The mass spectrometer was operated in the positive ion mode, and the ionspray needle, orifice and ring electrodes were maintained at 5000, 55 and 300 V, respectively. Nitrogen was used separately as collision, turboion-spray, nebulizer and curtain gas. The turboion-spray gas was heated to 350°C to assist in desolvation. For all Q1 scans, the mass spectrometer was scanned from m/z 50 to 750 in 0.25 m/z steps with 0.9 ms dwell time. For the MS/MS experiments, the collision energy was optimized between 35–50 eV for each set of experiments. All LC separations were performed by using a 100 × 2 mm × 5 μm Keystone Betasil C-18 column and Shimadzu LC-10-AS pumps, an SCL-10A controller and an SIL-10A autoinjector. The mobile phase, which consisted of 25 mM ammonium acetate at pH 4 (with acetic acid) (Solvent A) and a binary gradient that was described previously (38), was introduced to the CE rings, as described for similar steroids by Tomer and Gross (39), and some of the proposed structures are given in Figure 3. The fragment ion at m/z 256 (C12H10O2N5+‡) also arises by the cleavage of the C-6–C-7 and C-9–C-10 bonds in the CE B-ring. This fragmentation was proposed previously to be a charge driven process (40), which is initiated by a charge site at the N-9 position of the purine ring system. The ion at m/z 257 is interesting because it is likely to be a radical ion (C12H11O2N5‡). Radical ions are usually not fragments of closed-shell species unless they are unusually stable. Therefore, we suggest the fragment is a protonated semiquinone (i.e., a distonic ion). The product-ion spectrum of the 4-OHE1-1-N3Ade [M + H]‡ also shows the doublet at m/z 256 and 257, which suggests that formation of a semiquinone radical ion is general. Further investigations of this characteristic and interesting radical ion are underway. The ion of m/z 136 is important because it is likely to be protonated Ade, which identifies the adduct as a modified Ade.

With the ion trap instrument, the very low energy collisional activation produces principally product ions by losses of small neutral molecules, and as a result, the spectra are less distinct. Prominent are ions of m/z 405, 404 and 387 resulting from

**Results and discussion**

**Synthesis and structure elucidation of 4-OHE2-1-N3Ade and 4-OHE1-1-N3Ade**

The adduct 4-OHE2-1-N3Ade was synthesized by 1,4-Michael addition of Ade to E2-3,4-Q (Figure 2). Reaction of E2-3,4-Q with dA did not generate 4-OHE2-1-N3Ade, because the N-3 position of dA was shielded by the deoxyribose.

The NMR spectrum of 4-OHE2-1-N3Ade clearly shows both α- and β-isomers due to rotational restriction of the N3Ade. This phenomenon was observed previously in the NMR spectrum of 4-OHE2-1-N7Gua (25). Nuclear Overhauser effect NMR (spectrum not shown) indicated that the β-isomer is the major isomer, and the ratio of α/β is approximately 45:55, as seen previously for the 4-OHE2-1-N7Gua adduct (25). While these isomers can be distinguished by NMR, they cannot be separated, because rotation is partially, but not completely, hindered. This was also found for 4-OHE2-1-N7Gua (25).

Characterization of the structure of the adduct was derived from the singlet chemical shift of the 2-H of the CE moiety at 6.14 and 6.17 p.p.m. (β- and α-isomer, respectively). The spectrum also showed the 2-H of the Ade moiety resonating at 7.72 (β) and 7.74 p.p.m. (α). These chemical shifts are shielded with respect to the corresponding proton signals in the parent Ade, because these protons in the N3Ade adduct are close to the aromatic ring of the estrogen moiety. The 8-H of the Ade moiety was observed at 8.13 (α) and 8.24 p.p.m. (β).

The presence of the signal of the NH2 group of Ade, exchangeable with D2O, at 6.30 (β) and 6.75 p.p.m. (α) indicated that adduction had not occurred at the NH2 group. Since this adduct was formed only with Ade, and not with dA, we conclude that the covalent bond linked the 1-position of the CE moiety with the N-3 of Ade (26–29).

Further confirmation of the structure of the adduct was achieved by tandem mass spectrometry (MS/MS) on both an ion trap and a quadrupole time-of-flight (QTOF) mass spectrometer. We present the product-ion spectra obtained on the QTOF because they are more informative. The parent ion [M + H]‡ at m/z 422 gives product ions of m/z 390 (loss of methanol presumably), 296, 282, 270 and 256/257 (Figure 3A). These ions are all generated by cleavage of the CE rings, as described for similar steroids by Tomer and Gross (39), and some of the proposed structures are given in Figure 3B. The fragment ion at m/z 256 (C12H10O2N5+‡) also arises by the cleavage of the C-6–C-7 and C-9–C-10 bonds in the CE B-ring. This fragmentation was proposed previously to be a charge driven process (40), which is initiated by a charge site at the N-9 position of the purine ring system. The ion at m/z 257 is interesting because it is likely to be a radical ion (C12H11O2N5‡). Radical ions are usually not fragments of closed-shell species unless they are unusually stable. Therefore, we suggest the fragment is a protonated semiquinone (i.e., a distonic ion). The product-ion spectrum of the 4-OHE1-1-N3Ade [M + H]‡ also shows the doublet at m/z 256 and 257, which suggests that formation of a semiquinone radical ion is general. Further investigations of this characteristic and interesting radical ion are underway. The ion of m/z 136 is important because it is likely to be protonated Ade, which identifies the adduct as a modified Ade.
the losses of NH$_3$, H$_2$O, and both of the small molecules, respectively. Following loss of H$_2$O, a sequential loss of methane, most probably involving the C-18 methyl group, gives the fragment ion at m/z 388. The fragment ion m/z 393 (C$_{22}$H$_{25}$O$_3$N$_4$) arises by a loss of neutral CH$_2$NH from the purine ring system, which probably involves the amino group at C-6 of Ade. A product-ion spectrum of the m/z 393 (MS$^3$ experiment on the ion trap) shows that sequential loss of H$_2$O to give the m/z 375 ion is the major process. Other important ions arising from collisional activation of the m/z 393 ion are at m/z 365 (C$_{20}$H$_{23}$O$_3$N$_4$), m/z 281 (C$_{15}$H$_{13}$O$_2$N$_4$) and m/z 229 (C$_{11}$H$_{9}$O$_2$N$_4$). At the high collision energy, ions of m/z 365, 281 and 229 become more important, and they are formed by cross-ring cleavages, as discussed above.

4-OHE$_1$-1-N$_3$Ade was synthesized, purified and analyzed in the same way. It is also formed as α- and β-isomers in approximately the same ratio. The product-ion spectrum of 4-OHE$_1$-1-N$_3$Ade ([M + H]$^+$ 420; Figure 3B), taken with the QTOF, shows the ions of 310, 296, 282, 270, 256/257 and 228. As for the product-ion spectrum of 4-OHE$_2$-1-N$_3$Ade, these ions are structurally significant because they are formed by cross-ring cleavages of the steroid ring. The m/z values for these ions do not shift for 4-OHE$_1$-1-N$_3$Ade and 4-OHE$_2$-1-N$_3$Ade, indicating that the ion must contain the nucleobase and the base must be located on the A ring of the steroid.

The product-ion spectrum taken with the ion-trap instrument shows that the preferred product ions are of m/z 403, 402, 391, 386, 385 and 373, which are formed by the losses of small molecules, as was described above for 4-OHE$_2$-1-N$_3$Ade. The m/z values of these products shift down by two m/z units with respect to those of the 4-OHE$_2$-1-N$_3$Ade, indicating they contain the CE moiety.

In the m/z range below 300, there is a series of fragments at m/z 296, 257, 229, 228 and 201 that were also observed in the product-ion spectrum of 4-OHE$_2$-1-N$_3$Ade (discussed above). Fragmentation of 4-OHE$_1$-1-N$_3$Ade gives, as expected, a fragment of m/z 136, confirming the presence of the Ade moiety, but the abundance of this ion is considerably lower than that seen with the QTOF.

**Formation of 4-OHE$_2$-DNA adducts in vitro**

E$_2$-3,4-Q was directly reacted with DNA, as well as 4-OHE$_2$ activated by horseradish peroxidase, lactoperoxidase, tyrosinase or phenobarbital-induced rat liver microsomes (Table I). In all of the reactions, the depurinating adducts

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**Fig. 3.** MS/MS of (A) 4-OHE$_2$-1-N$_3$Ade and (B) 4-OHE$_1$-1-N$_3$Ade. The structures of the product ions are speculative and are given to show the utility of the fragmentation.
Table I. Reaction of E2-3,4-Q and enzyme-activated 4-OHE2 with DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Depurinating adducts, mmol/mol DNA-P*</th>
<th>Stable adducts, μmol/mol DNA-P*</th>
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<tr>
<td></td>
<td>4-OHE2-1-N7Gua</td>
<td>4-OHE2-1-N3Ade</td>
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<tr>
<td>E2-3,4-Q</td>
<td>186</td>
<td>196</td>
</tr>
<tr>
<td>4-OHE2</td>
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<td>Horseradish peroxidase</td>
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<td>Lactoperoxidase</td>
<td>363</td>
<td>208</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>Phenobarbital-induced rat liver microsomes</td>
<td>125</td>
<td>133</td>
</tr>
</tbody>
</table>

*These results are the average of two reactions, which differed by ≤10%.

Table II. Catechol estrogen metabolites and thioether conjugates detected in ACI rat mammary glands treated by intramamillary injection with E2-3,4-Q or 4-OHE2

<table>
<thead>
<tr>
<th>Conjugate/metabolite</th>
<th>pmol/g tissue*</th>
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<tbody>
<tr>
<td></td>
<td>E2-3,4-Q</td>
</tr>
<tr>
<td>4-OHE1</td>
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</tr>
<tr>
<td>4-OHE2</td>
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<tr>
<td>2-OCH3E2</td>
<td>-</td>
</tr>
<tr>
<td>E2</td>
<td>72</td>
</tr>
</tbody>
</table>

*These results are the average of two experiments, which differed by ≤10%.

4-OHE2-1-N7Gua and 4-OHE2-1-N3Ade constituted >99.9% of the adducts detected, and the level of stable adducts was <0.1 μmol/mol DNA-P. Approximately equal amounts of the two depurinating adducts were observed, except with activation by laccoperoxidase, which resulted in more N7Gua than N3Ade adduct. Tyrosinase yielded the lowest level of adducts, and microsomes gave about half the amounts detected from reaction of the quinone or 4-OHE2 activated with a peroxidase.

The HPLC detections of the adducts were confirmed by LC/MS/MS using a triple quadrupole mass spectrometer and selected reaction monitoring. We followed the fragmentation of the [M + H]+ ions of m/z 422 (Ade adduct) to give ions of m/z 136, 229 and 257 corresponding, respectively, to Ade (C9H8N3), C11H11N2O and C12H11N2O2, and the fragmentation of the [M + H]+ ions of m/z 438 (Gua adduct) to give ions of m/z 152, 272 and 312 corresponding, respectively, to Gua (C9H8N3O), C11H10N2O3 and C15H14N2O3. In the absence of internal standards, we decided not to quantify the amounts of the adducts by mass spectrometry.

These results confirm that oxidation of CE can be obtained by both peroxidases and cytochrome P450, and the oxidized material does react with DNA in vitro and cause depurination.

Estrogen metabolites, conjugates and depurinating adducts in mammary tissue

Female ACI rats were treated by intramamillary injection of 4-OHE2 or E2-3,4-Q for 1 h, and the mammary tissue was excised and analyzed for estrogen metabolites and conjugates or for estrogen–DNA adducts. Hexane was used to extract lipids from the mammary tissue. To extract estrogen metabolites and conjugates, the mammary tissue was suspended in 50 mM ammonium acetate, pH 5.0, with 2 mg/ml ascorbic acid to minimize oxidation of the estrogen derivatives. The extracts were passed through a Bond-Elut Certify II cartridge with non-polar and strong anion exchange properties. Untreated tissue samples spiked with 250 pmol of standard analytes/g tissue were used to determine the percentage of recovery of the various compounds. Recovery of estrogens, CE and their methoxy derivatives was >90%, whereas recovery of thioether conjugates and depurinating DNA adducts was 60–90%.

In the mammary tissue treated with E2-3,4-Q, the major metabolic event was reduction to 4-OHE2 (Table II). The amount corresponded to ~40% of that detected when the mammary tissue was treated with 4-OHE2 itself (see below). A significant amount of 4-OCH3E2 was detected. In addition, a small amount of E2 was unexpectedly detected, although E2 was not detected in the mammary tissue from control rats treated with DMSO.

The amounts of thioether conjugates were substantial, and the major ones were 4-OHE2-2-SG and 4-OHE2-2-Cys. Small amounts of 4-OHE2-2-NAcCys were also obtained. The conversion to estrone derivatives was very limited, and they consisted of 4-OHE1 and traces of 4-OHE1-2-Cys.

When the mammary tissue was treated with 4-OHE2, methylation to form 4-OCH3E2 was somewhat greater than oxidation to the corresponding quinone, as evidenced by 4-OHE2-2-SG, 4-OHE2-2-Cys and a small amount of 4-OHE2-2-NAcCys. Unexpectedly, E2, as well as 2-OHE2 and 2-OCH3E2, were detected following treatment with 4-OHE2. We do not know how these metabolites are formed, but we can speculate that induction of aromatase occurred, producing E2 and, thus, the small amount of 2-OHE2 metabolites.

We attempted to validate the detections of the conjugates with LC/MS/MS on an ion-trap mass spectrometer interfaced to capillary HPLC. Although the data suggest the presence of these materials, their amounts were too low in all cases to afford convincing product-ion spectra.

To analyze depurinating DNA adducts, mammary tissue was Soxhlet-extracted with chloroform/methanol. The extracts were first fractionated on preparative HPLC, and then the adduct-containing fractions were analyzed by HPLC with a CoulChem electrochemical detector.

In the group of rats treated with E2-3,4-Q, both the 4-OHE2-1-N7Gua and 4-OHE2-1-N3Ade adducts were detected, and in lesser amount the corresponding estrone adducts (Table III). Approximately equal amounts of N7Gua and N3Ade adducts...
were detected. The level of stable DNA adducts was <0.1% of the total adducts.

When the rats were injected with 4-OHE2, the same conversion of E2 to E1 was observed as with E2-3,4-Q. Slightly more N7Gua adducts (139 μmol/g tissue) were detected than N3Ade adducts (112 μmol/g tissue). The level of stable DNA adducts was <0.1% of the total adducts. The amount of total adducts obtained after treatment with the proximate metabolite 4-OHE2 was ~40% greater than that observed in the rats treated with the ultimate metabolite E2-3,4-Q. This is presumably due to the high reactivity of the quinone, which can react indiscriminately with various cellular molecules. In contrast, the 4-OHE2 can be oxidatively metabolized to E2-3,4-Q in regions close to the DNA.

We also conducted validation experiments with LC/MS/MS on the capillary HPLC/ion-trap mass spectrometer for three samples. For one sample, we were able not only to detect [M + H]^+ ions of the Ade and Gua adducts for E1 and E2, but also to obtain product-ion spectra that agree with the reference spectra of the Ade adducts (Figure 4). The product-ion spectra were obtained at sufficient collision energy to produce principally the losses of water, ammonia and water plus ammonia (Figure 4) but insufficient energy to give the ions in the mid mass range. The analysis of the two other samples showed successful detections of [M + H]^+ ions but gave product-ion spectra that were too weak to make a completely convincing validation. We now know that the QTOF is the preferred instrument for detection of these adducts because, as discussed above and seen in Figure 3, the spectra are considerably more structurally informative. At the time of analysis, however, we had not acquired the QTOF instrument.

**Conclusions**

The synthesis of 4-OHE1(E2)-1-N3Ade from E1(E2)-3,4-Q and Ade has allowed us to demonstrate that this adduct is also formed by reaction of E2-3,4-Q or enzymatically activated 4-OHE2 with DNA. In general, this adduct is formed in roughly the same amount as 4-OHE2-1-N7Gua both in vitro and in rat mammary gland in vivo. Detection of GSH conjugates in rat mammary tissue after treatment with 4-OHE2 or E2-3,4-Q indicates a competition between reaction of

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**Table III.** Catechol estrogen-DNA depurinating adducts detected in ACI rat mammary glands treated intramammillary with E2-3,4-Q or 4-OHE2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4-OHE2-1-N7Gua</th>
<th>4-OHE1-1-N7Gua</th>
<th>4-OHE2-1-N3Ade</th>
<th>4-OHE1-1-N3Ade</th>
<th>Stable adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2-3,4-Q</td>
<td>66</td>
<td>24</td>
<td>61</td>
<td>20</td>
<td>&lt;0.1% of total</td>
</tr>
<tr>
<td>4-OHE2</td>
<td>111</td>
<td>28</td>
<td>63</td>
<td>49</td>
<td>&lt;0.1% of total</td>
</tr>
<tr>
<td>Control (DMSO)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*These results are the average of two experiments, which differed by ≤10%.

*Not detected.

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**Fig. 4.** MS/MS of 4-OHE1-1-N3Ade formed in rat mammary gland.
E1(E2)-3,4-Q with GSH or DNA. The N3Ade and N7Gua depurinating adducts constitute >99% of the total adducts formed in vitro and in vivo. The N3Ade adducts, which are released from DNA instantaneously, generate mutations by error-prone repair (22,23). In contrast, the N7Gua adducts, which depurinate with a half-life of ~3 h (41), do not induce mutations, presumably because the slow rate of depurination allows the cell to repair correctly the DNA damage (22).

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References


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DNA adducts of E2-3,4-Q formed in vitro and in vivo