The Greater Reactivity of Estradiol-3,4-quinone vs Estradiol-2,3-quinone with DNA in the Formation of Depurinating Adducts: Implications for Tumor-Initiating Activity

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Received August 17, 2005

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Chem. Res. Toxicol. 2006, 19, 164−172

Strong evidence supports the idea that specific metabolites of estrogens, mainly catechol estrogen-3,4-quinones, can react with DNA to become endogenous initiators of breast, prostate, and other human cancers. Oxidation of the catechol estrogen metabolites 4-hydroxyestradiol (4-OHE2) and 2-OHE2 leads to the quinones, estradiol-3,4-quinone (E2-3,4-Q) and estradiol-2,3-quinone (E2-2,3-Q), respectively. The reaction of E2-3,4-Q with DNA affords predominantly the depurinating adducts 4-OHE2-1-N3Ade and 4-OHE2-1-N7Gua, whereas the reaction of E2-2,3-Q with DNA yields the newly synthesized depurinating adduct 2-OHE2-6-N3Ade. The N3Ade adducts are lost from DNA by rapid depurination, while the N7Gua adduct is lost from DNA with a half-life of ~3 h at 37 °C. To compare the relative reactivity of E2-3,4-Q and E2-2,3-Q, the compounds were reacted individually with DNA for 0.5−20 h at 37 °C, as well as in mixtures (3:1, 1:1, 1:3, and 5:95) for 10 h at 37 °C. Depurinating and stable adducts were analyzed. In similar experiments, the relative reactivity of 4-OHE2 and 2-OHE2 with DNA was determined after activation by lactoperoxidase, tyrosinase, prostaglandin H synthase (PHS), or 3-methylcholanthrene-induced rat liver microsomes. Starting with the quinones, the levels of depurinating adducts formed from E2-3,4-Q were much higher than that of the depurinating adduct from E2-2,3-Q. Similar results were obtained with lactoperoxidase or tyrosinase-catalyzed oxidation of 4-OHE2 and 2-OHE2, whereas with activation by PHS or microsomes, a relatively higher amount of the depurinating adduct from E2-2,3-Q was detected. These results demonstrate that the E2-3,4-Q is much more reactive with DNA than E2-2,3-Q. The relative reactivities of E2-3,4-Q and E2-2,3-Q to form depurinating adducts correlate with the carcinogenicity, mutagenicity, and cell-transforming activity of their precursors, the catechol estrogens 4-OHE2 and 2-OHE2. This is essential information for understanding the cancer risk posed by oxidation of the two catechol estrogens.

Introduction

The initial failure to demonstrate that estrogens induce mutations in bacterial and mammalian test systems (1−6) resulted in the classification of estrone (E1) and estradiol (E2) as epigenetic carcinogens that function mainly by stimulating abnormal cell proliferation via estrogen receptor-mediated processes (3, 7−12). The stimulated cell proliferation can result in more accumulation of genetic damage leading to carcinogenesis (9, 10, 12).

Compelling strong evidence has resulted in a new paradigm of cancer initiation by estrogens. Discovery that specific oxidative metabolites of estrogens can react with DNA (13−16) led to and supported the hypothesis that estrogen metabolites can become endogenous chemical carcinogens by generating mutations (17−19) that can lead to initiation of cancer. The initiating mechanism can occur in hormone-dependent and -independent tissues.

Catechol estrogens, 2-hydroxyestrone (estradiol) [2-OHE2(E2)] and 4-OHE2(E2), are among the major metabolites of E1 and E2. If these metabolites are oxidized to the electrophilic catechol estrogen quinones, they may react with DNA. The 4-catechol estrogens are carcinogenic in Syrian golden hamsters, as well as CD-1 mice (1, 20, 21), whereas the 2-catechol estrogens are not carcinogenic in the hamsters (1, 20) and are borderline carcinogens in CD-1 mice (21).

4-OHE2(E2) is easily oxidized to catechol estrogen-3,4-quinones [E1(E2)-3,4-Q], which react with DNA to form predominantly depurinating adducts (13−16). These adducts generate apurinic sites that may lead to cancer-initiating mutations (17−19), which transform cells (22−25), thereby initiating cancer.

To determine the DNA adducts of E1(E2)-3,4-Q, standard adducts were synthesized by reaction of the quinones with deoxyguanosine (dG), deoxyadenosine (dA), and the nucleobase Ade (16, 26). The reaction of E1(E2)-3,4-Q with dG afforded the depurinating adduct 4-OHE1(E2)-1-N7guanine (Gua) by 1,4-Michael addition (26). The reaction of E1(E2)-3,4-Q with dA did not produce any adduct. However, the reaction of these quinones with Ade resulted in the formation of 4-OHE1(E2)-1-N3Ade by 1,4-Michael addition (16). The rationale for the formation of N3Ade adducts is described in the Results and Discussion section.

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obtained (26). Reaction of E1(E2)-2,3-Q with dA afforded 2-OHE1(E2)-6-N6 Ade (Figure 1) and with dG yielded 2-OHE1(E2)-6-N2 dG (26). In these reactions, E1(E2)-2,3-Q did not react as quinones, but as their tautomers, the E1(E2)-2,3-Q methide (Figure 1). The electrophilic C-6 of the quinone methide reacted with the exocyclic amino group of dA or dG via 1,6-Michael addition to yield the N6 dA (Figure 1) and N2 dG adducts, which retain the deoxyribose moiety and are referred to as stable adducts because they remain in DNA unless repaired.

In this article, we report the synthesis of the N3Ade depurinating adduct obtained by reaction of E1(E2)-2,3-Q with Ade. We also report a study of the competitive reaction between estradiol-3,4-quinone (E2-3,4-Q) and estradiol-2,3-quinone (E2-2,3-Q) with DNA, as well as the metabolic oxidation of mixtures of 4-OHE2 (4-hydroxyestradiol) and 2-OHE2 to their quinones by selected enzymes in the presence of DNA to form DNA adducts. These results will be discussed in relation to the mechanism of tumor initiation by estrogens.

**Materials and Methods**

**Caution:** 2-OHE2, 4-OHE2, E2-2,3-Q, and E2-3,4-Q are hazardous chemicals and were handled according to NIH guidelines (27).

**Chemicals, Reagents, and Enzymes.** 2'-Iodoxybenzoic acid (IBX) was synthesized from 2'-iodobenzoic acid as described (28). 2-OHE2 and 4-OHE2 were synthesized by reacting E2 with IBX and then separating the mixture of 2-OHE2 and 4-OHE2 by HPLC. 4-OHE2-1-N3Ade and 4-OHE2-1-N7Gua were synthesized by published procedures (16, 26). MnO2, 2'-iodobenzoic acid, oxone, ascorbic acid, ammonium acetate, formic acid, sodium phosphate, adenine (Ade), DMSO-d6, CH3CN (HPLC grade), H2O2, lactoperoxidase (LP, from bovine milk), NADPH, methemoglobin, and tyrosinase (from mushrooms) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). dG and calf thymus DNA were purchased from USB (Cleveland, OH). Prostaglandin H synthase (PHS) and arachidonic acid were purchased from Cayman Chemical (Ann Arbor, MI), and CH3OH was purchased from Merck KGaA (Darmstadt, Germany). 3-Methylcholanthrene (MC)-induced rat liver microsomes were prepared as described earlier (29), containing 40 mg protein/mL, with 11.3 nmol cytochrome P450/mg protein.
Bond Elute Certify II SPE cartridges were purchased from Varian (Palo Alto, CA).

Activated MnO₂ was prepared as previously described (30), by treating concentrated aqueous KMnO₄ with aqueous MnSO₄ solution kept at 90 °C, until a slight excess of KMnO₄ was present, as indicated by the pink coloration of the suspension.

Instrumentation. 1. UV. The UV spectra were obtained during HPLC by using a photodiode array detector (PDA, Waters 996, Milford, MA) for all synthesized compounds. HPLC separations were monitored at 260 and 280 nm.

2. NMR. NMR spectra were recorded on a Varian Unity-Inova 500 instrument operating at a resonance frequency of 499.8 MHz for ¹H and 125.6 MHz for ¹³C spectra at 25 °C. Samples were dissolved in 600 μL of DMSO-d₆ and referenced to the solvent signals at 2.5 ppm for ¹H and 39.7 ppm for ¹³C. All two-dimensional (2D) experiments were performed by using the standard Varian software (VNMR v6.1c). For 2D experiments, relaxation delays of 1.2–2 s were used; 128–512 τᵣ increments and 2048 complex data points in τᵣ were recorded for a spectral width of 8000 Hz in two dimensions. ¹H-¹H correlations were recorded using correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) experiments. The TOCSY experiment was performed in the states TPPI mode with a MLEV17 spin lock at 10 kHz field strength. In pulsed field gradient ¹H-¹C heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) sequences, delays were optimized for coupling constants around 140 and 8 Hz, respectively. One-dimensional nuclear Overhauser (NOE) experiments were recorded in difference mode by subtracting one spectrum with irradiation on resonance from another without irradiation with a relaxation delay of 5 s.

3. Mass Spectrometry. Fast atom bombardment tandem mass spectrometry (FAB-MS) was conducted at the Nebraska Center for Mass Spectrometry (University of Nebraska-Lincoln) using a MicroMass (Manchester, England) AutoSpec high-resolution magnetic sector mass spectrometer. The instrument was equipped with an orthogonal acceleration time-of-flight serving as the second mass spectrometer. Xenon was admitted to the collision cell at a level to provide an orthogonal acceleration time-of-flight serving as the second mass spectrometer. Xenon was admitted to the collision cell at a level to provide a 996 PDA by using a linear gradient of 10% CH₃ CN in H₂O.

Preparative HPLC was conducted on a Luna-2 C-18 column (5 μm, 120 A, 250 mm × 250 mm, Phenomenex, Torrance, CA) on a Waters 600E solvent delivery system equipped with a 996 PDA by using a linear gradient of 10% CH₃ CN:CH₃ OH:0.1 M HCOONH₄ (pH 3.7), 15:5:80; and B, CH₃ CN:CH₃ OH:0.1 M HCOONH₄ (pH 3.7), 50:20:30. The linear gradient changed from 100% A to 90% B in 50 min. The serial arrays of 12 coulometric electrodes were set at potentials of −10, 100, 150, 210, 270, 330, 390, 450, 510, 570, and 630, and 690 mV. The 50 μL injections were carried out on a Phenomenex Luna-2 C-18 column (5 μm, 120 A, 4.6 mm × 250 mm) at 1 mL/min. The system was controlled, and the data were acquired and processed using the CoulArray software package.

Synthesis of Standard Adducts. To a stirred solution of 2-OHE₂, or 2-OHE (0.2 mmol) in 5 mL of CH₃ CN at −40 °C, activated MnO₂ (2 mmol) was added under an argon atmosphere (Figure 1). After 10 min, the yellowish green solution was filtered through a 0.45 μm Gelman acrodisc directly into a flask containing a solution of Ade (1.2 mmol) in CH₃ COOH/H₂O (1:1 v/v, 6 mL). The reaction mixture was stirred overnight at room temperature. It was evaporated to dryness, and the residue was redissolved in dimethylformamide/CH₃ OH (1:1, 3 mL) and then subjected to preparative HPLC for purification of the adducts.

1. 2-OHE₂-6-N₃Ade. Yield 43%. UV: λmax 212, 263 nm. ¹H NMR (ppm): 8.93 (s, 1H, OH, exchangeable with D₂O), 8.77 (s, 1H, OH, exchangeable with D₂O), 8.17 (s, 1H, 8-H-Ade), 7.47 (s, 1H, 2-H-Ade), 7.20 (s, 2H, NH₂-Ade, exchangeable with D₂O), 6.80 (s, 1H, 1-H), 6.30 (s, 1H, 4-H), 5.65 (d, 1H, J = 4.0 Hz, 6-H), 4.45 (s, 1H, 17β-OH, exchangeable with D₂O), 3.49 (t, 1H, J = 8.5 Hz, 17α-H), 2.20 (dd, 1H, J₁ = 10.5, J₂ = 1.5 Hz), 2.11–2.13 (m, 1H), 1.96 (d, 1H, J = 14.0 Hz), 1.75–1.91 (m, 4H), 1.47–1.52 (m, 2H), 1.38–1.40 (m, 1H), 1.13–1.28 (m, 3H), 0.61 (s, 3H, CH₃). ¹C NMR (ppm): 156.0, 152.2, 149.3, 145.8, 144.0, 140.1, 132.7, 122.8, 118.8, 116.0, 112.6, 80.0, 51.3, 48.8, 43.3, 43.1, 36.6, 33.9, 33.5, 29.9, 25.8, 22.7, 11.3. FAB-MS [M⁺ + H⁺]: 422.2170 calculated for C₂₃H₂₈N₅O₃; observed, 422.2192. 2. 2-OHE₂-6-N₆Ade. Yield 9%. UV: λmax 214, 277 nm. ¹H NMR (ppm): 9.25 (br.s, 2H, OH, exchangeable with D₂O), 8.23 (s, 1H, 8-H-Ade), 8.07 (s, 1H, 2-H-Ade), 7.73 (br.s, 1H, 6-NH⁻Ade, exchangeable with D₂O), 6.67 (s, 1H, 1-H), 6.55 (s, 1H, 4-H), 5.42 (br.s, 1H, 6-H), 4.40 (s, 1H, 17β-OH, exchangeable with D₂O), 3.53 (t, 1H, J = 8.5 Hz, 17α-H), 2.09–2.19 (m, 1H), 1.95–2.04 (m, 1H), 1.73–1.93 (m, 4H), 1.29–1.58 (m, 4H), 1.06–1.25 (m, 3H), 0.74 (s, 3H, CH₃). FAB-MS [M⁺ + H⁺]: 422.2170 calculated for C₂₃H₂₈N₅O₃; observed, 422.2197.

4. 2-OHE₂-6-N₆Ade. Yield 41%. UV: λmax 214, 264 nm. ¹H NMR (ppm): 8.97 (s, 1H, OH, exchangeable with D₂O), 8.77 (s, 1H, OH, exchangeable with D₂O), 8.17 (s, 1H, 8-H-Ade), 7.58 (s, 1H, 2-H-Ade), 7.19 (s, 2H, NH₂-Ade, exchangeable with D₂O), 6.78 (s, 1H, 1-H), 6.27 (s, 1H, 4-H), 5.70 (d, 1H, J = 4.5 Hz, 6-H), 5.45 (s, 1H, 17β-OH), 2.20 (dd, 1H, J₁ = 10.5, J₂ = 1.5 Hz, 1-H), 1.96–2.13 (m, 1H), 1.75–1.91 (m, 4H), 1.47–1.52 (m, 2H), 1.38–1.40 (m, 1H), 1.13–1.28 (m, 3H), 0.61 (s, 3H, CH₃). FAB-MS [M⁺ + H⁺]: 420.2025 calculated for C₂₃H₂₈N₅O₃; observed, 420.2036.

Preparation of Quinones. 2-OHE₂ (2.5 mg, 8.7 μmol) was dissolved in 500 μL of CH₃ CN and stirred at −40 °C. Then, activated MnO₂ (7.5 mg, 86.2 μmol) was slowly added. After 15 min, the yellowish green solution was filtered through a Gelman acrodisc. Because of the instability of E₂-3-O, the quinone in CH₃ CN was directly used in an experiment as quickly as possible. 4-OHE₂ (2.5 mg, 8.7 μmol) was dissolved in 500 μL of CH₃ CN and stirred at 0 °C. Then, activated MnO₂ (7.5 mg, 86.2 μmol) was slowly added. After 20 min, the solution was filtered through a Gelman acrodisc and an equal amount of DMSO was added. The CH₃ CN was evaporated in a high vacuum rotavapor using dry ice and acetone in the condenser. After evaporation of CH₃ CN, the quinone in DMSO was used for the experiments. The final
Concentration of quinone (either individually or together) in the reaction was 0.87 mM in DMSO.

Covalent Binding of E2-2,3-Q or E2-3,4-Q to DNA. Freshly prepared E2-2,3-Q and E2-3,4-Q (0.87 mM total concentration in 0.5 mL of DMSO) individually, as well as in mixtures (Figure 2), were mixed with DNA (3 mM in 0.067 M sodium potassium phosphate buffer, pH 7.0) and incubated at 37 °C for different time periods (0.5, 1, 2, 3, 5, 10, 15, and 20 h). At the indicated times, DNA was precipitated with two volumes of ethanol, and the supernatant, containing depurinating adducts, was concentrated to 1 mL under low pressure, extracted by using a vac-Elute system, and finally analyzed for depurinating adducts by HPLC with an electrochemical detector, as described above. The levels of depurinating adducts were determined by comparing peak heights with known adduct standards. Precipitated DNA from a 1 mL aliquot from each experiment was separated for 32P-postlabeling analysis of stable adducts after purification of the DNA (31).

Covalent Binding of 2-OHE2 and 4-OHE2 to DNA. Mixtures containing different relative concentrations of 2-OHE2 and 4-OHE2 (0.87 mM total concentration, Figures 3–7) were incubated with DNA in the presence of different enzymes, including tyrosinase, LP, PHS, and MC-induced rat liver microsomes. The reaction volume in each experiment was 10 mL. In the tyrosinase experiments, the mixture containing 3 mM calf thymus DNA in 0.067 M sodium—potassium phosphate buffer (pH 7.0), 0.87 mM 2-OHE2 or 4-OHE2 (2.5 mg in 500 µL of DMSO), and 1 mg of enzyme (2577 units) was incubated at 37 °C for 2 or 10 h. For the LP-catalyzed reaction, the mixture containing 3 mM calf thymus DNA, 0.87 mM 2-OHE2 or 4-OHE2 (2.5 mg/µL of DMSO), H2O2 (0.5 mM), and 1 mg of enzyme (97 units) was incubated at 37 °C for 2 or 10 h. For PHS-catalyzed reactions, the mixture containing 3 mM DNA, 0.87 mM 2-OHE2 or 4-OHE2 (2.5 mg in 500 µL of DMSO), 1 mL of methemoglobin (2.95 mg/mL in 75 mM KH2PO4, pH 7.5), 1 mL of arachidonic acid (50 mM), and 800 µL of PHS (400 units) was incubated at 37 °C for 2 or 10 h. For the microsome-catalyzed reaction, the 10 mL mixture containing 3 mM DNA in 150 mM Tris-HCl (pH 7.5), 150 mM KCl, 5 mM MgCl2, 0.87 mM 2-OHE2 or 4-OHE2 (2.5 mg in 500 µL of DMSO), 10 mg of microsomal protein (40 mg/mL), and NADPH (0.6 mM) was incubated at 37 °C for 2 or 10 h. A 1 mL aliquot from each reaction mixture was removed, and the DNA was analyzed for stable DNA adducts (31). From the remaining incubation mixture, DNA was precipitated with 2 volumes of ethanol, and the supernatant was used for the analysis of depurinating adducts, as described above. Control reactions were carried out under identical conditions with either no enzyme or no cofactor.

Results and Discussion

Synthesis and Characterization of Standard Depurinating Adducts. The reaction of E2-2,3-Q with Ade yielded the major depurinating adduct, 2-OHE2-6-N3Ade (43%), and a minor product, 2-OHE2-6-N6Ade (9%) (Figure 1). The reaction of E2-2,3-Q with dA yielded only 2-OHE2-6-N6dA (Figure 1) (26). The reaction with dA or Ade does not occur in ring A of the estrogen, because of the weak electrophilicity of positions 1 and 4. However, after tautomeration of the E1(E2)-2,3-Q to E1(E2)-2,3-Q methide, the reaction takes place at C-6 via 1,6-Michael addition. The N3Ade adduct could not be obtained by reaction of E2-2,3-Q with dA because electrophilic attack of the C-6 of E2-2,3-Q methide at the nucleophilic N-3 group of dA is hindered by the presence of the deoxyribose moiety bound to the adjacent N-9 in dA. The dA also cannot react with E2-3,4-Q (3), hexestrol-3′,4′-quinone (32), and polycyclic aromatic hydrocarbons (33–36) to form N3Ade adducts. With DNA, instead, N3Ade adducts are formed with estrogens (16,32) and aromatic hydrocarbons (37,38) and rapidly lost by depurination, because the configuration of the deoxyribose moiety in DNA renders the N-3 group available.

The 1H NMR spectrum of 2-OHE2-6-N3Ade showed two singlets at 8.93 and 8.77 ppm for the two hydroxy groups in the catechol moiety, which were confirmed by D2O exchange. The two single proton integration signals at 8.17 and 7.47 ppm were assigned to H-8 (Ade) and H-2 (Ade), respectively, based on similar values in other N3Ade adducts (16,32,34–36). In fact, the significant upfield shift of the H-2 (Ade) proton at 7.47 ppm, as compared to H-2 in unsubstituted Ade, strongly indicates that this proton is shielded by the aromatic ring of the
estrogen moiety. This corroborates substitution at N-3 in the proposed structure. The two signals at 6.80 and 6.30 ppm were assigned as the aromatic protons H-1 and H-4, respectively, based on 1D and 2D NMR studies, which ruled out the possibility of the substitution of Ade in the aromatic ring (at C-1 and C-4) of E2. Furthermore, the spectrum showed the doublet of one proton at 5.65 ppm, which correlated to a methine group at 51.3 ppm in the HSQC experiment. These 1H and 13C chemical shifts suggested a substitution of the base at the C-6 position of E2. This is in accordance with the earlier observation that E1-2,3-Q undergoes tautomerization to its quinone methide (26); thus, the value of 5.65 ppm was assigned to the H-6 of E2. Furthermore, the resonance of this proton shows COSY correlation with aliphatic protons of the steroid skeleton at 1.76 ppm, assigned as H-7 in COSY and TOCSY experiments. In addition, H-7 showed correlations in the COSY spectrum with two other protons, which give a mutual multiplet at 1.74–1.87 ppm. The broad signal at 7.20 ppm, exchangeable with D2O, two other protons, which give a mutual multiplet at 1.74–1.87 ppm. The broad signal at 7.20 ppm, exchangeable with D2O, corresponding to two protons in the 1H NMR spectrum, excluded the possibility of attachment of the estrogen moiety at the NH2 of Ade. This was further confirmed by an NOE experiment, in which irradiation of the signal at δ 6.30 (H-4) produced a strong effect on the signal at δ 7.47 (H-2, Ade) and vice versa. These extensive NMR studies unequivocally establish the structure of the compound as 2-OHE2-6-N3Ade. Although the nucleophilic attack of the N-3 position of Ade can take place at either face α or β at C-6 of the estrogen quinone methide, we obtained only one product, in which the attachment occurred at the α face (from below the estrogen plane). Attack at the upper face β appears to be hindered by the methyl group at C-13.

The 1H NMR spectrum of 2-OHE2-6-N6Ade showed a broad singlet integrated as two protons at 9.25 ppm for the two hydroxy groups, exchangeable with D2O. Three singlets of one proton each at 8.23, 8.07, and 7.73 ppm were assigned to the H-8, H-2, and NH of the Ade moiety, respectively. Two singlets at 6.67 and 6.55 ppm were assigned to the H-1 and H-4 protons of the E2 moiety, respectively; one broad singlet at 5.42 ppm (one proton) for the H-6 of the E2 moiety strongly suggests substitution at the C-6 position; one singlet at 4.40 ppm for the C-17 hydroxy group and one triplet at 3.53 ppm for the C-17 proton in the estrogen moiety were also present. The presence of a broad singlet at 7.53 ppm corresponding to one proton suggests that the 6-NH is substituted in the proposed structure. The 1H NMR spectra of 2-OHE1-6-N3Ade and 2-OHE1-6-N6Ade are very similar to those of 2-OHE2-6-N3Ade and 2-OHE2-6-N6Ade, except that the former two show no signals for the C-17 hydroxy group and C-17 proton. Thus, the structure of 2-OHE1-6-N3Ade and 2-OHE1-6-N6Ade is also established. All four compounds showed the desired exact [M + H]+ in their FAB-MS.

Relative Reactivity of E2-3,4-Q and E2-2,3-Q with DNA. Different concentrations of E2-3,4-Q were reacted with 3 mM DNA to examine the saturation level in the formation of depurinating adducts (4-OHE2-1-N3Ade and 4-OHE2-1-N7Gua). The level of 0.87 mM E2-3,4-Q was found to be saturating for measurement of the rate of reaction between E2-3,4-Q and DNA (data not shown).

Incubations were conducted at 37 °C for 10 h because the N7Gua adduct depurinates slowly. When E2-3,4-Q was reacted with DNA for 10 h, the adducts 4-OHE2-1-N3Ade and 4-OHE2-1-N7Gua were detected at approximately equal levels of 130–140 μmol/mol DNA-P (data not shown).

The slow depurination of 4-OHE2-1-N7Gua is clearly seen in Figure 2, in which equal amounts of E2-3,4-Q and E2-2,3-Q were reacted with DNA and the depurinating adducts were analyzed at various time points. The binding of E2-3,4-Q to DNA and depurination of the N3Ade adduct was complete within 1 h, suggesting that depurination was instantaneous. In contrast, the depurination of the N7Gua adduct had a half-life of approximately 3 h at 37 °C and was complete in 10 h. This slow loss of deoxyribose was previously observed in the reaction of E2-3,4-Q with dG to form the N7Gua adduct (39). The adduct 2-OHE2-6-N3Ade was formed at a much lower level, 4 μmol/mol DNA-P, and depurination was also immediate (Figure 2). The level of stable adducts determined by the 32P-postlabeling technique was <0.5% of the total adducts formed (data not shown).
This study was further delineated by determining the level of the three depurinating adducts formed at different ratios of E2-3,4-Q and E2-2,3-Q (Figure 3). The overwhelming abundance of the depurinating N7Gua and N3Ade adducts formed by E2-3,4-Q is observed at all ratios of quinones. The levels of the three depurinating adducts were similar only with 5% E2-3,4-Q and 95% E2-2,3-Q. The level of stable adducts formed in these mixtures ranged from 0.1% of total adducts with 100% E2-3,4-Q to 1% of total adducts with 100% E2-2,3-Q (data not shown).

Relative Reactivity of Enzyme-Activated 4-OHE2 and 2-OHE2 with DNA. When mixtures of 2-OHE2 and 4-OHE2 at different ratios were reacted with DNA in the presence of tyrosinase, the depurinating adducts from 4-OHE2 were the most abundant. The levels of the depurinating adducts were similar only with 5% 2-OHE2 and 95% 4-OHE2 present (Figure 4). The levels of stable adducts detected in the reaction mixtures were approximately the same, which ranged from 0.1 to 0.7% of total adducts (data not shown). Similar relative amounts of the three depurinating adducts were obtained when LP was used to activate the catechol estrogens (Figure 5). The amounts of stable adducts formed in the mixtures were similar, ranging from 0.2 to 0.8% of total adducts (data not shown). With activation by PHS, relatively lower amounts of adducts were formed from the 4-OHE2, as compared to activation by tyrosinase or LP. The level of adducts from 2-OHE2 remained, instead, about the same (Figure 6). In this experiment, with 95% 2-OHE2 and 5% 4-OHE2, the levels of the two depurinating adducts from 4-OHE2 were about half the amount of 2-OHE2-6-N3Ade. The level of stable adducts was a little higher, and they represented 0.5% of total adducts with 95–100% 2-OHE2 (data not shown).

The activation of 4-OHE2 was relatively lower when MC-induced rat liver microsomes were used for activation (Figure 7). In fact, 100% 4-OHE2 yielded only 15–20 µmol/mol DNA-P of 4-OHE2-1-N7Gua and 4-OHE2-1-N3Ade. The 2-OHE2-6-
N3Ade was obtained in relatively larger amounts. In fact, at a ratio of 75% 2-OHE2 to 25% 4-OHE2, similar amounts of the three depurinating adducts were detected (Figure 7), and with 95% 2-OHE2, the 2-OHE2-6-N3Ade was observed to be present in about a 3-fold higher amount than the two adducts formed from 4-OHE2. With the microsomes, the amount of stable adducts ranged from 0.12 μmol/mol DNA-P with 100% 4-OHE2 to 1.57 μmol/mol DNA-P with 100% 2-OHE2, representing 0.3 (with 4-OHE2) to 11.4% (with 2-OHE2) of total adducts (data not shown). This 10-fold increase in the amount of stable adducts formed from 2-OHE2 as compared to 4-OHE2 is similar to results previously obtained when E2-3,4-Q or E2-2,3-Q with Ade forms 4-OHE2-1-N3Ade (16) or 2-OHE2-6-N3Ade, respectively. The reaction of E2-3,4-Q with DNA yields 4-OHE2-1-N3Ade, which is rapidly depurinated, and 4-OHE2-1-N7Gua, which is depurinated slowly, with a half-life of about 3 h (Figure 2). The final amounts of the two adducts are very similar (130–140 μmol/mol DNA-P). The reaction of E2-2,3-Q with DNA affords 2-OHE2-6-N3Ade, which depurinates immediately. The maximum amount of this adduct is 12 μmol/mol DNA-P (Figure 3).

**Conclusions**

The reaction of E2-3,4-Q with dG forms the depurinating adduct 4-OHE2-1-N7Gua (13, 16), whereas the reaction of E2-3,4-Q or E2-2,3-Q with Ade forms 4-OHE2-1-N3Ade (16) or 2-OHE2-6-N3Ade, respectively. The reaction of E2-3,4-Q with DNA yields 4-OHE2-1-N3Ade, which is rapidly depurinated, and 4-OHE2-1-N7Gua, which is depurinated slowly, with a half-life of about 3 h (Figure 2). The final amounts of the two adducts are very similar (130–140 μmol/mol DNA-P). The reaction of E2-2,3-Q with DNA affords 2-OHE2-6-N3Ade, which depurinates immediately. The maximum amount of this adduct is 12 μmol/mol DNA-P (Figure 3).
When mixtures of E₂-3,4-Q and E₂-2,3-Q (3:1, 1:1, 1:3, and 5:9) react with DNA, the levels of 4-OHE₂-1-N7Gua and 4-OHE₂-1-N3Ade decrease with increasing amounts of E₂-2,3-Q but are always much greater than the level of 2-OHE₂-6-N3Ade (Figure 3). When mixtures of 4-OHE₂ and 2-OHE₂ are activated by tyrosinase, LP, or PHS (Figures 4–6) in the presence of DNA, various levels of the adducts are obtained, but the levels of the N3Ade and N7Gua depurinating adducts are much greater than the level of 2-OHE₂-6-N3Ade. The level of 2-OHE₂-6-N3Ade is higher than the levels of 4-OHE₂-1-N3Ade and 4-OHE₂-1-N7Gua only with PHS and 95% 2-OHE₂. With rat liver microsomes, relatively more depurinating adducts are formed from 2-OHE₂, with equal amounts of all three depurinating adducts being observed at 25% 4-OHE₂ and 75% 2-OHE₂ (Figure 7). The above data are essential for predicting the relative amounts of depurinating adducts found in vivo when various ratios of 2-OHE₂ and 4-OHE₂ are formed during the oxidative metabolism of estrogens.

The greater reactivity of E₂-3,4-Q with DNA in forming depurinating adducts of Ade and Gua is in line with the carcinogenic effect of 4-OHE₂ in inducing kidney tumors in hamsters (1, 20) and uterine adenocarcinomas in CD-1 mice (21). The much lower reactivity of E₂-2,3-Q with DNA to form 2-OHE₂-6-N3Ade also correlates with the lack of induction of kidney tumors by 2-OHE₂ in hamsters (1, 20) and the much weaker ability to induce uterine adenocarcinomas in CD-1 mice (21). Furthermore, in breast tissue from women with breast carcinoma, 4-OHE₂(E₂) were 3.5 times more abundant than 2-OHE₂(E₂) and were four times higher than in breast tissue from women without breast cancer (41, 42).

When cultured human breast epithelial (MCF-10F) cells, which are estrogen receptor-negative, are treated with physiological levels of 4-OHE₂, cellular transformation occurs. An approximately million times higher concentration of 2-OHE₂ is needed to transform these cells (22, 23). Transformation occurs even in the presence of the antiestrogen ICI-182,780 (24), indicating that transformation does not proceed through estrogen receptor-mediated events (25). In fact, specific mutations are induced by treatment of MCF-10F cells with 4-OHE₂ (25).

Acknowledgment. This research was supported by Grant P01 CA49210 from the National Cancer Institute and Grant DAMD17-03-10229 from the Department of Defense Breast Cancer Research Program. Core support at the Epplle Institute was provided by Grant P30 CA36727 from the National Cancer Institute.


TX050229Y