# The Equine Estrogen Metabolite 4-Hydroxyequilenin Causes DNA Single-Strand Breaks and Oxidation of DNA Bases in Vitro

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Premarin (Wyeth-Ayerst) is the estrogen replacement treatment of choice and continues to be one of the most widely dispensed prescriptions in North America. In addition to endogenous estrogens, Premarin contains unsaturated equine estrogens, including equilenin [1,3,5(10),6,8-estrapentaen-3-ol-17-one]. In previous work, we showed that the equilenin metabolite 4-hydroxyequilenin (4-OHEN) can be autoxidized to 4-OHEN-*o*-quinone which readily entered into a redox couple with the semiquinone radical catalyzed by NAD(P)H, P450 reductase, or quinone reductase, resulting in generation of reactive oxygen species [Shen, L., Pisha, E., Huang, Z., Pezzuto, J. M., Krol, E., Alam, Z., van Breemen, R. B., and Bolton, J. L. (1997) *Carcinogenesis* **18**, 1093−1101]. As oxidative damage to DNA by reactive oxygen species generated by redox active compounds has been proposed to lead to tumor formation, we investigated whether 4-OHEN could cause DNA damage. We treated $\lambda$ phage DNA with 4-OHEN and found that extensive single-strand breaks could be obtained with increasing concentrations of 4-OHEN as well as increasing incubation times. If scavengers of reactive oxygen species are included in the incubations, DNA could be completely protected from 4-OHEN-mediated damage. In contrast, NADH and $CuCl_2$ enhanced the ability of 4-OHEN to cause DNA single-strand breaks presumably due to redox cycling between 4-OHEN and the semiquinone radical generating hydrogen peroxide and ultimately copper peroxide complexes. We also confirmed that 4-OHEN could oxidize DNA bases since hydrolysis of 4-OHEN-treated calf thymus DNA and HPLC separation with electrospray MS detection revealed oxidized deoxynucleosides, including 8-oxodeoxyguanosine and 8-oxodeoxyadenosine. Our data suggest that DNA single-strand breaks and oxidation of DNA bases by 4-OHEN could contribute to the carcinogenic mechanism(s) of equine estrogens.

## Introduction

Oxidative damage to DNA is thought to play a significant role in spontaneous mutagenesis, cancer, aging, and other human pathologies (*1*, *2*). Redox active xenobiotics as well as endogenous compounds can spontaneously or enzymatically generate reactive oxygen species such as superoxide. Dismutation of superoxide gives hydrogen peroxide, which through metal-catalyzed Fenton chemistry can form the ultimate oxidant, the hydroxyl radical that could be responsible for DNA oxidation. Alternatively, transition metals such as iron and copper can react with hydrogen peroxide, generating metal peroxide complexes which can cause DNA strand breaks and oxidation of DNA bases (*3*, *4*). The most thoroughly studied oxidized base is 8-oxoguanine (8-oxo-G)[1] since a highly sensitive HPLC method has been developed to detect the corresponding deoxynucleoside (8-oxo-dG) using electrochemical detection (*5*). However, several other oxidized bases could be formed (Figure 1), including 8-oxoadenine (8-oxo-A), 2-oxoadenine (2-oxo-A), thymine glycol, 5-hydroxymethyluracil, and 5-formyluracil (*4*). Subsequent replication of the oxidatively damaged DNA can result in double mutations (*6*) and single-base substitutions (*7*).

The excessive production of reactive oxygen species in breast cancer tissue has been linked to metastasis of tumors in women with breast cancer (*8*). The source of reactive oxygen species has been suggested to be the result of redox cycling of the endogenous estrogen metabolites, the catechol estrogens (*9*, *10*). Peroxidase/P450-catalyzed oxidation of these catechols gives *o*-quinones,

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[1] Abbreviations: dN, 2′-deoxynucleoside; dG, 2′-deoxyguanosine; dA, 2′-deoxyadenosine; dC, 2′-deoxycytosine; 8-oxo-dG, 8-oxodeoxyguanosine; 8-oxo-dA, 8-oxodeoxyadenosine; 2-oxo-dA, 2-oxodeoxyadenosine; 8-oxo-G, 8-oxoguanine; 8-oxo-A, 8-oxoadenine; 2-oxo-A, 2-oxoadenine; 2-OHE, 2-hydroxyestrone, 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one; 4-OHE, 4-hydroxyestrone, 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one; 4-OHEN, 4-hydroxyequilenin, 3,4-dihydroxy-1,3,5(10),6,8-estrapentaen-17-one; estrone, 3-hydroxy-1,2,5(10)-oestratrien-17-one; equilenin, 1,3,5(10),6,8-estrapentaen-3-ol-17-one; equilin, 1,3,5(10),7-estratetraen-3-ol-17-one; ERT, estrogen replacement therapy; P450, cytochrome P450.

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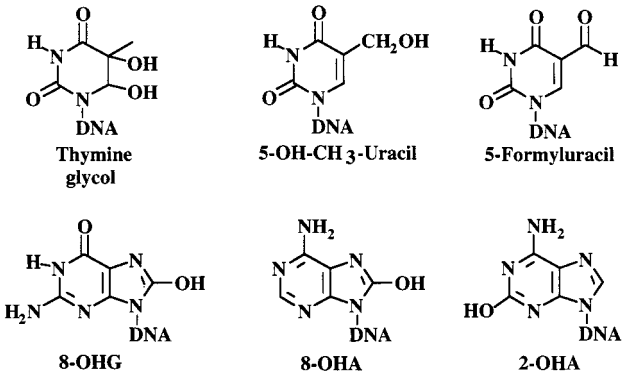


**Figure 1.** Potential DNA oxidation products.

which have previously been implicated as the ultimate carcinogens. Redox cycling between the *o*-quinones and their semiquinone radicals generates superoxide, hydrogen peroxide, and ultimately reactive hydroxyl radicals, which cause oxidative cleavage of the phosphate−sugar backbone as well as oxidation of the purine/pyrimidine residues of DNA (*11*). In support of this mechanism, various free radical toxicities have been reported in hamsters treated with 17$\beta$-estradiol, including DNA single-strand breaks (*11*, *12*), lipid peroxidation (*13*), 8-oxo-dG formation (*10*), and chromosomal abnormalities (*14*, *15*). Moreover, in hamsters treated with 17$\beta$-estradiol, antioxidants such as BHA (*16*) or ascorbate (*17*) significantly reduced the incidence of tumors compared to estrogen treatment alone. Finally, elevated levels of oxidized dG and dA have been detected in the breast tissue of cancer patients compared to controls, consistent with a major role of reactive oxygen species in cancer initiation and/or progression (*18*, *19*). Increased levels of 8-oxo-dG in particular have been associated with a predictive significance for breast cancer risk assessment (*20*).

The equine estrogen equilenin [1,3,5(10),6,8-estrapentaen-3-ol-17-one] or its 17$\beta$-hydroxylated analogue makes up 5% of the most widely prescribed estrogen replacement formulation, Premarin (Wyeth-Ayerst), yet there is very little information on the metabolism of these estrogens either in animal models (*21*−*24*) or in women. It is known that treating hamsters for 9 months with either estrone, equilin and equilenin, or sulfatase-treated Premarin resulted in 100% tumor incidences and abundant tumor foci (*24*). We previously synthesized the major metabolite of equilenin, 4-hydroxyequilenin (4-OHEN, Figure 2), and examined how aromatization of the B ring affects the formation and reactivity of the equilenin quinoids (*25*). Unlike the endogenous catechol estrogens, 4-OHEN rapidly autoxidized to 4-OHEN-*o*-quinone which readily entered into a redox couple with the semiquinone radical catalyzed by NAD(P)H, P450 reductase, or quinone reductase (Figure 2; *25*). Significant oxygen consumption was also detected, consistent with in vitro models that have shown that 4-OHEN-*o*-quinone increases the amount of oxidative damage to DNA by 50% compared to control levels (*26*). Finally, we showed that the 4-OHEN-semiquinone radical forms very unusual cyclic adducts with deoxynucleosides and DNA which may represent one mechanism for equilenin carcinogenesis (*27*, *28*). We report here the relative ability of 4-OHEN to cause DNA single-strand breaks as well as the oxidized bases resulting from reaction of 4-OHEN with DNA under redox cycling conditions.

## Materials and Methods

**Chemicals and Reagents. Caution:** *All catechol estrogens were handled in accordance with NIH guidelines for the Laboratory Use of Chemical Carcinogens (29).* All chemicals were purchased from Aldrich (Milwaukee, WI), Fisher Scientific (Itasca, IL), or Sigma (St. Louis, MO) unless stated otherwise. 4-OHEN was synthesized by treating equilin with Fremy's salt as described previously (*26*, *30*) with minor modifications (*27*). 8-Oxo-dA was prepared as described previously (*31*). Briefly, 2′-deoxyadenosine (500 mg) was treated with 0.5 M sodium acetate containing a saturated bromine/water solution, giving 8-bromo-2′-deoxyadenosine (40% yield): $^1$H NMR (DMSO-$d_6$) $\delta$ 2.18−2.23 (m, 1H, 2′-H), 3.22−3.37 (m, 1H, 2′-H), 3.48−3.52 (m, 1H, 5′-H), 3.62−3.68 (m, 1H, 5′-H), 3.90 (m, 1H, 4′-H), 4.48 (m, 1H, 3′-H), 5.27−5.37 (m, 2H, 3′,5′-OH), 6.30 (m, 1H, 1′-H), 7.55 (s, 2H, 6-$NH_2$), 8.11 (s, 1H, 2-H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 37.8, 62.9, 72.0, 87.2, 89.1, 120.5, 127.5, 150.7, 153.2, 155.9; UV ($CH_3$-OH) 214, 264 nm; electrospray MS (positive ion, relative intensity) *m*/*z* 352 ($MH^+$, 100%), 354 ($M + 2 + H^+$, 100%). 8-Bromo-2′-deoxyadenosine was converted to 8-oxo-dA as follows. DMSO (15 mL), benzyl alcohol (5 mL), and sodium metal (170 mg, 7.39 mmol) were combined, and the solution was stirred until the sodium dissolved. 8-Bromo-2′-deoxyadenosine was added to the solution, and the mixture was stirred for 26 h at 70 °C. The reaction was neutralized with glacial acetic acid, and the solid was precipitated with 450 mL of diethyl ether. After filtration, the precipitate was washed with diethyl ether and methanol and then purified by flash chromatography with $CH_3OH/CHCl_3$/acetone (1:5:1) as the eluant. Removal of solvent en vacuo gave 8-oxo-2′-deoxyadenosine (28% yield): $^1$H NMR (DMSO-$d_6$) $\delta$ 2.00 (m, 1H, 2′-H), 2.96 (m, 1H, 2′-H), 3.45 (m, 1H, 5′-H), 3.59 (m, 1H, 5′-H), 3.81 (s, 1H, 4′-H), 4.38 (s, 1H, 3′-

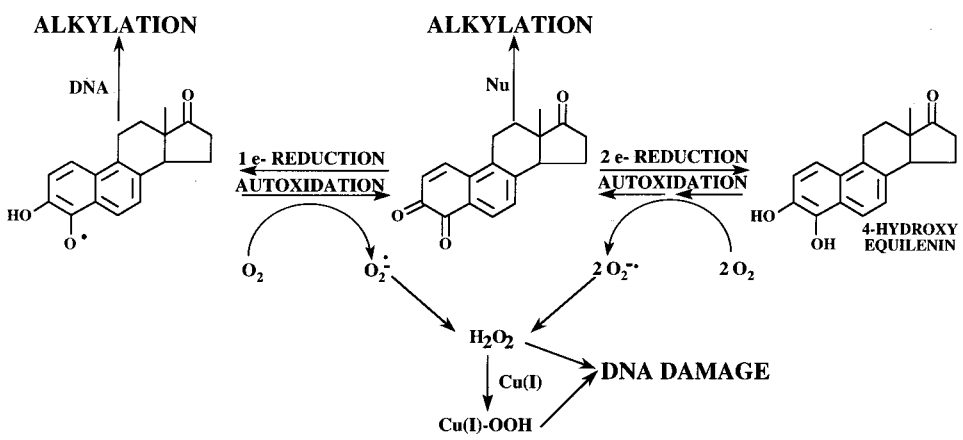


**Figure 2.** Proposed model for 4-OHEN toxicity and DNA damage.

H), 5.14−5.21 (m, 2H, 3′,5′-OH), 6.11−6.36 (m, 1H, 1′-H), 6.64 (s, 2H, 6-$NH_2$), 8.01 (s, 1H, 2-H), 10.46 (broad s, 1H, 8-OH); $^{13}C$ NMR (DMSO-$d_6$) $\delta$ 37.7, 63.3, 72.7, 83.3, 88.5, 104.3, 146.8, 147.9, 150.8, 152.6; UV ($CH_3OH$) 210, 270 nm; electrospray MS (positive ion, relative intensity) *m*/*z* 268 ($MH^+$, 100%); electrospray MS/MS (positive ion, relative intensity) of the *m*/*z* 268 ion 152 (M − 116, 100%), 135 (20%), 125 (30%), 108 (20%), similar to what has been reported in the literature (*32*).

**DNA Single-Strand Breaks Produced by 4-OHEN.** Typical reaction conditions were as follows. DNA [$\lambda$ phage (Sigma), 0.5 $\mu$g/$\mu$L] was incubated with 4-OHEN (0.5 mM) in 50 mM potassium phosphate buffer (10 $\mu$L) at pH 7.4 and 20 °C. In all cases, the appropriate controls were included in the same experiment. The solutions were mixed and incubated for 30 min. The DNA was denatured in boiling water for 3 min in the presence of 5 $\mu$L of DNA sequencing stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol). The denatured DNA was immediately subjected to electrophoresis on a 0.7% agarose gel with 0.5 $\mu$g/mL ethidium bromide in 40 mM TRIS-acetate buffer containing 1 mM EDTA. The DNA was visualized under UV irradiation at 312 nm. After electrophoresis for 1 h, the agarose gel was photographed with a Polaroid 3000 camera.

It was observed that an excess of GSH (10-fold relative to the 4-OHEN concentration) could completely protect $\lambda$ phage DNA from 4-OHEN-mediated damage. As a result, we exploited this effect to determine the time required for 4-OHEN to cause DNA single-strand breaks. 4-OHEN (2 mM) was mixed with $\lambda$ phage DNA (0.5 $\mu$g/$\mu$L) in 80 $\mu$L of 50 mM potassium phosphate buffer (pH 7.4) at 20 °C. At various time points, aliquots (10 $\mu$L) were removed and combined with 10 mM GSH. After 30 min, the aliquots were denatured and subjected to electrophoresis on a 0.7% agarose gel as described above.

**4-OHEN-Mediated Oxidation of DNA Bases.** Calf thymus DNA (Sigma) (50 mg) was dissolved in 10 mL of TE buffer [10 mM Tris, 5 mM NaCl, and 1 mM EDTA (pH 7.8)] and repeatedly precipitated with 70% ethanol/water to remove all impurities. A 1 mL solution of DNA (2 mg/mL) in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA was incubated with 4-OHEN (2.0 mM) and ampicillin (100 $\mu$g/mL) for 3 h at 37 °C. At the conclusion of the incubation, the DNA was precipitated with 70% ethanol/water and washed with three 5 mL portions of 70% ethanol. The precipitated DNA was hydrolyzed to deoxynucleosides using the following literature procedures with minor modifications (*33*, *34*). Briefly, the DNA was redissolved in 1 mL of DNase buffer [50 mM sodium acetate, 2 mM $CaCl_2$, and 10 mM $MgCl_2$ (pH 6.5)] and incubated with DNase I (20 units/mg of DNA) for 20 h at 37 °C. Then 50 $\mu$L of 0.5 M Tris buffer (pH 9) and snake venom phosphodiesterase (0.013 unit/mg of DNA) were added, and the mixture was incubated at 37 °C for 4 h. This treatment resulted in complete hydrolysis to the deoxynucleoside level. The DNA hydrolysates were neutralized with 1 M HCl, and aliquots (80 $\mu$L) were analyzed by HPLC with an Ultrasphere ODS column (4.6 mm × 250 mm, Beckman) with a flow rate of 1.0 mL/min on a Shimadzu LC-10A gradient HPLC apparatus equipped with a SIL-10A auto injector, a SPD-M10AV UV/VIS photodiode array detector, and a SPD-10AV UV detector set at 280 nm. The HPLC mobile phase consisted of 1.0% $CH_3OH$ in water which was increased to 5.0% $CH_3OH$ in 10 min, to 15% $CH_3OH$ in another 24 min, and to 90% $CH_3OH$ in the last 6 min of the run. For LC/MS analysis (electrospray, positive ion), aliquots (25 $\mu$L) were analyzed directly by HPLC on a Hewlett-Packard (Palo Alto, CA) 1090L gradient HPLC apparatus equipped with a photodiode array UV/vis absorbance detector set at 230−350 nm and a 5989B MS Engine quadrupole mass spectrometer. Under these conditions, the retention times and $MH^+$ ions of the oxidized deoxynucleosides were as follows: 8-oxo-dG, 26 min, $MH^+$, 284; and 8-oxo-dA, 37 min, $MH^+$, 268.

**8-Oxo-dG Analysis.** Deoxyguanosine (dG) and 8-oxo-dG concentrations in the DNA hydrolysates were determined by HPLC with UV and electrochemical detection, respectively, as described previously (*5*). The deoxynucleosides were separated as described above using a HPLC apparatus (Shimadzu LC-10AS) coupled to UV photodiode array and electrochemical (EC) detectors (ESA CoulArray). For EC detection, the channels, numbered 1−4, were set at the following values: 200, 290, 380, and 430 mV, respectively. Aliquots (50 mL) of the DNA hydrolysate were injected onto a Partisil 5 $\mu$m ODS-3 reverse phase analytical column [4.6 mm × 250 mm (i.d.); Whatman, Clifton, NJ] that was maintained at 25 °C. The mobile phase contained 4 mM citric acid, 8 mM ammonium acetate, 10% methanol, and 20 mg/L EDTA, saturated with helium (ultrapure grade), at pH 4 with a flow rate of 1 mL/min. The 8-oxo-dG was eluted with a retention time of 27.5 min. Quantification of 8-oxo-dG was obtained from EC channel 2 (290 mV) analysis. Quantitations of dC, dT, dG, and dA were performed by UV absorption and monitored at 254 nm, and they were eluted with retention times of 9.6, 18, 19.9, and 30.5 min, respectively. Concentrations of 8-oxo-dG were expressed relative to the concentrations of dG detected by UV absorbance at 254 nm. A Student's *t* test was used for statistical analysis of the data.

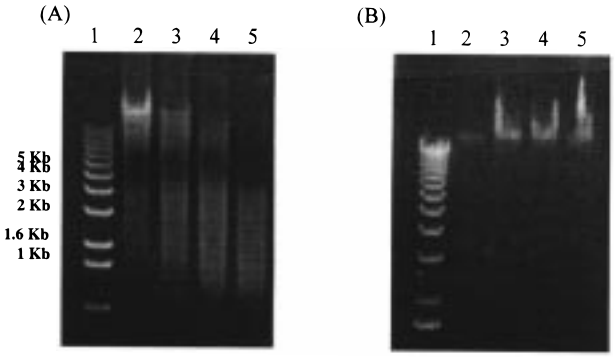


**Figure 3.** Dose dependence of 4-OHEN-mediated DNA damage. (A) Agarose gel electrophoresis of $\lambda$ DNA (0.5 $\mu$g/$\mu$L) incubated at 20 °C with various concentrations of 4-OHEN: lane 1, molecular weight markers (1−12 kb); lane 2, 0.4 $\mu$L of acetone; lane 3, 4-OHEN (0.1 mM); lane 4, 4-OHEN (0.5 mM); and lane 5, 4-OHEN (2 mM). (B) As in panel A using DNA which had not been denatured.

**Instrumentation.** HPLC experiments were performed on the above-mentioned Shimadzu LC-10A gradient HPLC system. Peaks were integrated with Shimadzu EZ-Chrom software and a 486-33 computer. UV spectra were measured with a Hewlett-Packard model 8452 photodiode array UV spectrophotometer, and $^1H$ NMR and $^{13}C$ NMR spectra were obtained with a Bruker Avance 300 spectrometer. Positive ion electrospray mass spectra were obtained using a Hewlett-Packard 5989B MS Engine quadrupole mass spectrometer equipped with a ChemStation data system and a high-flow pneumatic nebulizer-assisted electrospray LC/MS interface. The mass spectrometer was interfaced to the above-mentioned Hewlett-Packard gradient HPLC system. The quadrupole analyzer was maintained at 120 °C, and unit resolution was used for all measurements. Nitrogen at a pressure of 80 psi was used for nebulization of the HPLC effluent, and nitrogen bath gas at 250 °C with a flow rate of 10 L/min was used for evaporation of the solvent from the electrospray system. The *m*/*z* range of 200−900 was scanned every 2 s during LC/MS. LC/MS/MS experiments were performed on a Micromass quattro II electrospray triple-quadrupole mass spectrometer. Samples were infused using a syringe pump in 50% methanol/water containing 1% acetic acid.

## Results

Figure 3 shows the effects of incubating increasing concentrations of 4-OHEN with $\lambda$ phage DNA. The damage is characteristic of single-strand breaks since no fragmentation was observed in similar experiments with double-stranded DNA which had not been denatured

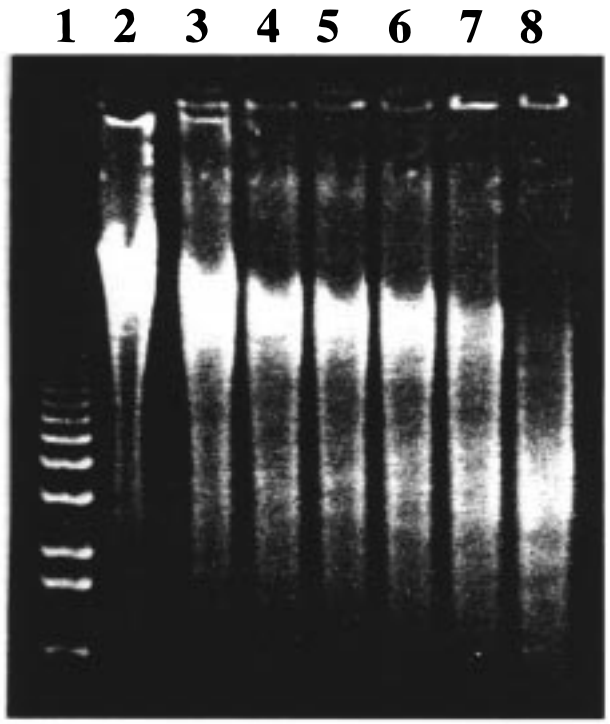


**Figure 4.** Time course for 4-OHEN-mediated DNA damage. Agarose gel electrophoresis of $\lambda$ DNA (0.5 $\mu$g/$\mu$L) incubated at 20 °C with 4-OHEN (1 mM). GSH (10 mM) was added at various times to quench 4-OHEN and prevent further DNA damage: lane 1, molecular weight markers (1−12 kb); lane 2, 0 min; lane 3, 1 min; lane 4, 3 min; lane 5, 6 min; lane 6, 10 min; lane 7, 20 min; and lane 8, 30 min.

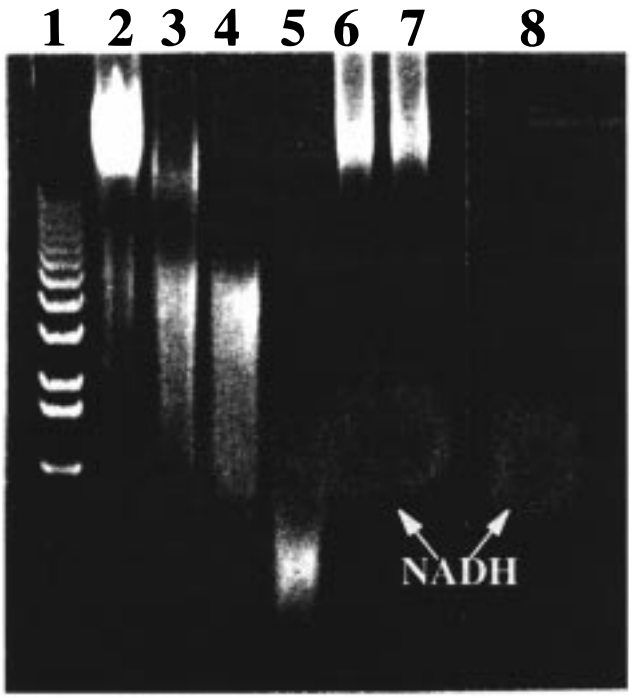


**Figure 5.** Effect of redox cycling on 4-OHEN-mediated DNA damage. Agarose gel electrophoresis of $\lambda$ DNA (0.5 $\mu$g/$\mu$L) incubated at 20 °C with various combinations of 4-OHEN, NADH, and/or $CuCl_2$: lane 1, molecular weight markers (1−12 kb); lane 2, 0.4 $\mu$L of acetone; lane 3, 4-OHEN (0.5 mM); lane 4, 4-OHEN (0.5 mM) and NADH (4 mM); lane 5, 4-OHEN (0.5 mM), NADH (4 mM), and $CuCl_2$ (0.1 mM); lane 6, NADH (4 mM) and $CuCl_2$ (0.1 mM); lane 7, NADH (4 mM); and lane 8, NADH (4 mM) alone.

prior to electrophoresis (Figure 3B). DNA in the vehicle-treated sample (Figure 3A, lane 2) is clearly visible, whereas increasing concentrations of 4-OHEN caused a dose-dependent disappearance of high-molecular weight (MW) DNA and an enhancement in DNA with fewer kilobases indicative of strand scission.

The effect of incubation time on the ability of 4-OHEN to damage DNA is shown in Figure 4. In this experiment, we took advantage of the observation that high concentrations of GSH can completely protect DNA from 4-OHEN-mediated damage since GSH can trap the 4-OHEN-*o*-quinone (J. L. Bolton et al., unpublished results). Although the resulting GSH conjugates also have the potential to redox cycle (*35*, *36*), the high concentration of GSH is much more effective at scavenging reactive oxygen species as compared to DNA and DNA remains intact. By varying the time of addition of GSH, we can determine the time course of 4-OHEN single-strand cleavage. Figure 4 shows that within 1 min of reaction with 4-OHEN a significant increase in DNA damage has occurred (Figure 4, lane 3). The extent of damage increases with time until the 30 min time point, where no intact DNA can be detected (Figure 4, lane 8).

To investigate the effects of 4-OHEN-mediated redox cycling, we incubated 4-OHEN with $\lambda$ phage DNA and NADH (Figure 5, lane 4). Increases in the amount of single-strand cleavage were observed relative to that for 4-OHEN alone (Figure 5, lane 3) or vehicle-treated DNA (Figure 5, lane 2). The addition of $CuCl_2$ (Figure 5, lane 5) almost completely destroyed the DNA likely due to formation of copper hydroperoxide complexes (*3*, *4*, *37*) which could result from reaction of copper with hydrogen peroxide generated by redox cycling of 4-OHEN. NADH and $CuCl_2$, or NADH alone, had no effect on DNA as shown in lanes 6 and 7, respectively. NADH does appear as a fluorescent spot in these control samples but not in lanes 3−5 since it is completely consumed by continuous reduction of 4-OHEN-*o*-quinone (*25*).

The ability of scavengers of reactive oxygen species to protect DNA from 4-OHEN-mediated damage is shown in Figure 6. Including azide (lane 4), mannitol (lane 5), and catalase (lane 6) which scavenge singlet oxygen, free hydroxyl radicals, and hydrogen peroxide respectively, completely protected DNA from single-strand breaks. In contrast, SOD could not protect the DNA from 4-OHEN. These data strongly suggest that singlet oxygen, hydroxyl radicals, and hydrogen peroxide play a major role in 4-OHEN-mediated DNA damage.

Determining the type of lesions on DNA bases caused by redox cycling of 4-OHEN was of interest. When calf thymus DNA was treated with 4-OHEN, hydrolyzed to deoxynucleosides, and analyzed by HPLC, chromatograms similar to the one in Figure 7B were obtained. Very significant increases in 8-oxo-dG levels were observed relative to that of the vehicle-treated control, i.e., 100-fold greater for the 4-OHEN-treated sample compared to the control (Table 1). Including NADH in the incubation further increased the 8-oxo-dG peak (Figure 7C and Table 1); however, additional oxidized deoxynucleosides were also observed, including 8-oxo-dA as well as some unidentified peaks. The identity of 8-oxo-dA was confirmed by co-injection of the synthesized standard and comparisons of LC/MS and UV analyses. By constructing a standard curve with the synthesized 8-oxo-dA, we have determined the amount of 8-oxo-dA formed to be 0.094 ± 0.008 8-oxo-dA/dA% which is

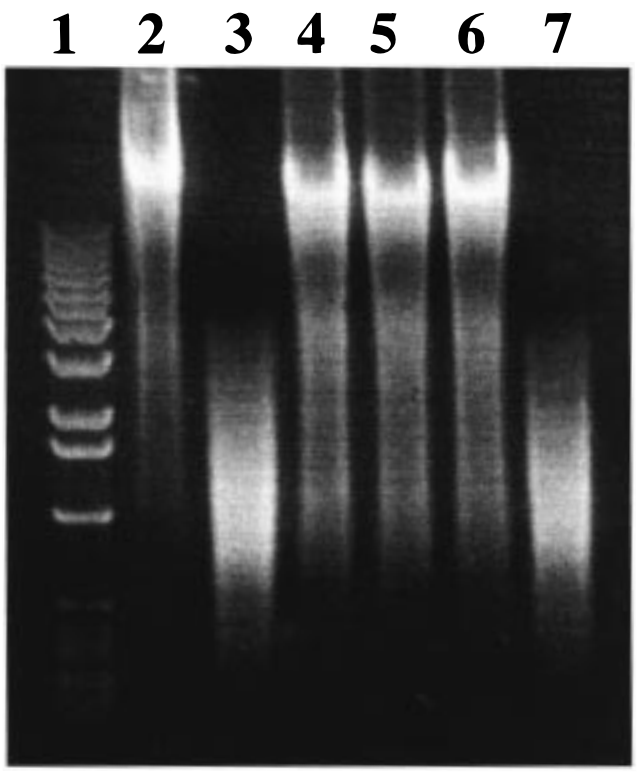


**Figure 6.** Effect of scavengers of ROS on 4-OHEN-mediated DNA damage. Agarose gel electrophoresis of $\lambda$ DNA (0.5 $\mu$g/$\mu$L) incubated at 20 °C with various combinations of 4-OHEN and Scavengers of ROS: lane 1, molecular weight markers (1−12 kb); lane 2, 0.4 $\mu$L of acetone; lane 3, 4-OHEN (2 mM); lane 4, 4-OHEN (2 mM) and azide (50 mM); lane 5, 4-OHEN (2 mM) and mannitol (50 mM); lane 6, 4-OHEN (2 mM) and catalase (5 units); and lane 7, 4-OHEN (2 mM) and SOD (5 units).

**Table 1. 4-OHEN-Mediated 8-Oxidation of Guanine Bases of DNA[a]**

| reaction conditions | 8-oxo-dG/dG% |
|---|---|
| acetone (40 $\mu$L/mL) | 0.005 ± 0.001[b] |
| NADH | 0.003 ± 0 |
| NADH and $CuCl_2$ | 0.067 ± 0.005 |
| 4-OHEN | 2.9 ± 0.4 |
| 4-OHEN and NADH | 3.7 ± 0.2[c] |
| 4-OHEN, NADH, and $CuCl_2$ | 3.8 ± 0.5[c] |

[a] Calf thymus DNA (2 mg/mL) was treated with 4-OHEN (2 mM) in phosphate buffer at pH 7.4 and 37 °C for 3 h. The DNA was precipitated, washed with ethanol, and hydrolyzed to deoxynucleosides. The hydrolysate was analyzed and its 8-OHdG content determined as described in Materials and Methods. The concentration of NADH was 3 mM, and that of $CuCl_2$ was 0.1 mM. The data represent an average ± SD of three determinations. [b] Average of two determinations. [c] Significantly different from that for 4-OHEN alone ($p < 0.05$).

considerably less than the amount of 8-oxo-dG produced (Table 1). The peak at 30 min was determined to be adenosine by comparison of the MS/MS daughter ion spectra with that of the authentic standard. The source of adenosine is unknown at present; however, it may come from degradation of NADH by copper peroxy radicals.

## Discussion

Our data clearly demonstrate that 4-OHEN is capable of causing DNA single-strand breaks and oxidative damage to DNA bases in addition to our previous reports which showed that 4-OHEN has the ability to covalently modify DNA (*27*, *28*). The electrophoretic mobility of double-stranded DNA was unaffected under similar reaction conditions which suggests that 4-OHEN causes single-strand breaks in DNA. Similarly, when 4-hydroxyestrone-*o*-quinone was incubated in MCF-7 cells, single-strand breaks in cellular DNA were also formed but double-strand DNA breaks were not detected (*12*). The damage is dose- (Figure 3) and time-dependent (Figure 4), could be increased in the presence of reducing agents, and reducing agents and $CuCl_2$ (Figure 5), and could be prevented in the presence of scavengers of singlet oxygen, hydroxyl radicals, and hydrogen peroxide (Figure 6). Copper is closely associated with DNA in the cell, particularly with guanine-rich regions (*38*). It has been reported that copper interacts with hydrogen peroxide, forming copper peroxide complexes as shown below (*3*, *4*), which are likely involved in the enhanced DNA damage observed in this study (adapted from ref *3*).

$$\mathrm{DNA{-}(Cu^{II})_2 + H_2O_2 \rightarrow DNA{-}(Cu^{I})_2 + O_2}$$

$$\mathrm{DNA{-}Cu^{I} + H_2O_2 \rightarrow DNA{-}Cu^{I}OOH}$$

The ability of copper to catalyze DNA damage has been previously reported for other hydroxylated aromatic compounds, including the endogenous catechol estrogens (*39*), hydroquinone (*37*), and the catechol metabolite of the antitumor agent etoposide (*40*). In addition, a recent report has shown that *o*-quinones formed from polycyclic aromatic hydrocarbon cause a significant amount of DNA strand scission in the presence of NADPH and $CuCl_2$ (*41*) which suggests that similarities in structure between 4-OHEN-*o*-quinone and these PAH metabolites may explain their proposed comparable cytotoxic and mutagenic mechanisms (*42*).

Treating calf thymus DNA with 4-OHEN, hydrolyzing the DNA to deoxynucleosides, and HPLC analysis revealed a substantial increase in 8-oxo-dG formation (100-fold). Supporting this, previous reports have shown that incubations with 4-OHEN-*o*-quinone, DNA, and hamster liver microsomes also enhanced 8-oxo-dG formation 2-fold relative to control samples (*26*). We believe that the substantial increases in 8-oxo-dG levels observed in this study are the result of starting from the catechol rather than from the *o*-quinone, since autoxidation of the catechol generates reactive oxygen species leading to 8-oxo-dG formation. Interestingly, when we included NADH in the incubations with 4-OHEN and DNA, 8-oxo-dA was observed (Figure 7C). These data are particularly intriguing since 8-oxo-A (*43*) and 8-oxo-G (*44*) are mutagenic lesions associated with a heightened cancer risk. These and other data are evidence for a mechanism of estrogen-induced tumor initiation by redox cycling of estrogen metabolites generating reactive oxygen species which damage DNA as shown in Figure 2.

We previously showed that 4-OHEN has the ability to form highly unusual cyclic adducts with deoxynucleosides and DNA (*27*, *28*). Three different types of adducts were formed, including unstable adenine adducts which readily depurinate, leaving apurinic sites on the DNA. Stable bulky adducts were also produced with guanine and cytosine which if formed in vivo must be repaired by nucleotide excision repair enzymes. In this investigation, we have shown that 4-OHEN also has the ability to oxidize DNA bases, forming a number of different mutagenic lesions. Neither DNA alkylation nor oxidation

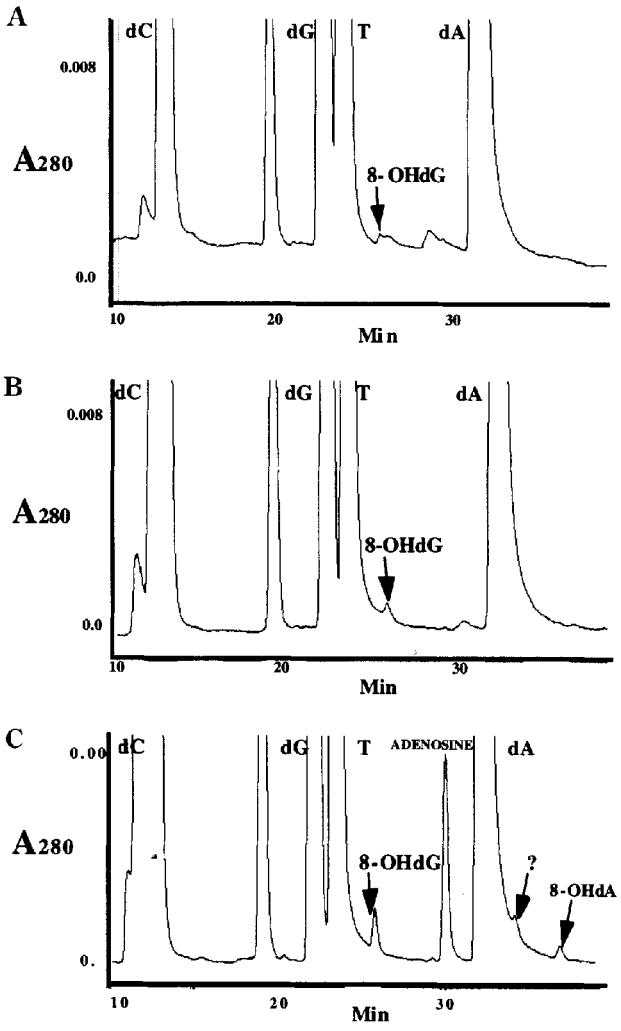


**Figure 7.** HPLC analysis of 4-OHEN-mediated oxidation of DNA. Calf thymus DNA (2 mg/mL) was treated with 4-OHEN (2 mM) in phosphate buffer at pH 7.4 and 37 °C for 3 h. The DNA was precipitated, washed with ethanol, and hydrolyzed to deoxynucleosides. The hydrolysate was analyzed by HPLC using a methanol/water mobile phase as described in Materials and Methods. (A) acetone (40 $\mu$L/mL), (B) 4-OHEN (2 mM), and (C) 4-OHEN (2 mM) and NADH (3 mM).

requires metabolic activation of 4-OHEN since this catechol readily autoxidizes. This is in contrast to the endogenous catechol estrogens which must have enzymatic or metal ion catalysis prior to formation of oxidized DNA bases (*23*) and strand breaks (*11*, *12*). Similarly, although 4-OHE and 2-OHE can alkylate DNA in vivo (*45*−*48*) and in vitro (*47*), they must be metabolized to *o*-quinones prior to alkylation.

Finally, we have shown that 4-OHEN can generate DNA single-strand breaks in a dose- and time-dependent manner as well as form a variety of mutagenic oxidized lesions on DNA bases. This is in addition to our previous reports of stable bulky adducts and apurinic sites which result from interaction of 4-OHEN with DNA (*27*, *28*). These in vitro experiments suggest that this equine estrogen metabolite has the ability to initiate an extensive assault on DNA, which could easily overwhelm cellular repair enzymes. It is not known whether 4-OHEN could alkylate and/or oxidize DNA in vivo; however, given the direct link between long-term, high-dose estrogen replacement therapy and increased risk for breast and endometrial cancer, it is crucial that the reactive intermediates formed from estrogen replacement formulations and their cellular targets be thoroughly explored.

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