Characterization of Semiquinone Free Radicals Formed from Stilbene Catechol Estrogens

AN ESR SPIN STABILIZATION AND SPIN TRAPPING STUDY

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Electron spin resonance spectroscopy has been used to detect, characterize, and to infer structures of o-semiquinones derived from stilbene catechol estrogens. Radicals were generated enzymatically using tyrosinase and were detected as their Mg²⁺ complexes. It is suggested that initial hydroxylation of stilbene estrogen gives a catechol estrogen in situ; subsequent two-electron oxidation of the catechol to the quinone, followed by reverse disproportionation, leads to the formation of radicals. Consistent with this mechanism, o-phenylenediamine, a quinone trapping agent, inhibits formation of o-semiquinones. A competing mechanism of radical production involves autoxidation of the catechol. Hydroxyl radicals are shown to be produced in this system via a mechanism involving reduction of iron and copper complexes by stilbene catechols. Possible differences in the reactivity of stilbene ortho- and para-semiquinones are discussed.

There is increasing evidence for metabolic activation of stilbene estrogens playing a critical role in the induction of tumors in experimental animals and humans exposed to these agents (1-9). In vivo studies using adult, fetal, and neonatal animals have shown that the carcinogen diethylstilbestrol (DES)1 undergoes extensive metabolism to a variety of products arising from side chain alterations, ring-hydroxylation (4, 5), and ring-oxidation to dihydrostilbestrol-4',4''-quinone.2 Administration of α-naphthoflavone, a well-known inhibitor of microsomal enzymes, has been shown to inhibit the development of tumors in Syrian hamsters undergoing treatment with estrogens (6). Ascorbic acid administration also reduces the tumor incidence in animals exposed to DES (7).

The exact nature of cytochrome P-450-mediated activation of stilbenes has not yet been fully clarified. However, redox cycling in vitro between DES and diethylstilbestrol-4',4''-quinone in the presence of microsomes and NADPH has been demonstrated (8). This redox cycling results in the generation of superoxide radicals (9), presumably by interaction of molecular oxygen with the semiquinone intermediate.

A free radical mechanism of induction of DNA damage by structurally diverse estrogens has been postulated (10) to play a role in renal tumorigenesis in Syrian hamsters. In this species, the production of identical sets of target organ-specific DNA adducts precedes the induction of renal cancer (10). This DNA damage is considered to be generated by unidentified endogenous electrophile(s) activated by estrogens, possibly via a free radical mechanism. The suspected role of free radicals in such DNA alterations has led to a heightened interest in estrogen semiquinones and their reactions with endogenous substances.

Free radical o-semiquinones have previously been identified by ESR spin stabilization after oxidation of 2- or 4-hydroxysteroidal (11, 12). More recently, this technique has also been applied in the identification of o-semiquinones formed from o-quinones bound covalently to peptides and proteins (13). o-Semiquinones from stilbene estrogens have not previously been identified, although such free radical species may play a role in the activation of the potent carcinogens HEX and (E,E)-DIES (14). These stilbene estrogens cannot be oxidized to bridged methide quinones and may therefore require initial o-hydroxylation. Support for the role of catechol and, possibly, catechol o-semiquinone formation in stilbene estrogen carcinogenesis comes from several observations, for example, the demonstrated formation of significant amounts of aromatic ring-hydroxylated or -methoxylated metabolites in the metabolism of HEX, DIES (15), or DES (4, 5) both in vitro and in vivo. In addition, in mammalian tissue culture systems, the extent of cell transformation induced by estrogens parallels the rate of catechol metabolite formation (16, 17). Finally, the lack of carcinogenic activity (18) of 2-fluoroestradiol, a compound of considerable hormonal potency (19), has been suspected to be due to decreased conversion of this modified steroid to catechol metabolites (20).

In this work, experiments were carried out to understand the mechanism of formation of oxyradicals and other radicals...
from metabolic redox cycling of estrogens. Identification of \( \alpha \)-semiquinones from metabolic redox cycling of estrogens. Identification of \( \alpha \)-semiquinones from Stilbene Catechol Estrogens 11015 from metabolic redox cycling of estrogens. Identification of \( \alpha \)-semiquinones formed during the tyrosinase-catalyzed oxidation of DES, DIES, and HEX and their deuterated analogs is reported. Their ESR spectral patterns are analyzed to facilitate in \( \textit{vitro} \) or \( \textit{in vivo} \) studies by ESR on the role of free radicals in hormonal carcinogenesis.

The tyrosinase/\( \mathcal{O}_2 \) system was chosen to generate the respective catechols \( \textit{in situ} \) because most of the catechols were very unstable and could not be isolated in pure form. Structures of the various estrogens and the catechol estrogens are shown in Scheme 1.

**MATERIALS AND METHODS**

**Chemicals**—DES, HEX, (E,E)-DIES, and tyrosinase were purchased from Sigma. (Z,Z)-DIES was synthesized as described previously (21). The preparation of \( 2,2,3',3'',5,5,5',5'' \)-octadeuteriodiethylstilbestrol, \( 3,3',5,5' \)-tetradeteriohexestrol, and \( 2,3',3'',5,5',5'' \)-hexadeuterio-Z,Z-dienestrol has been described by Liehr and Ballatore (22). Dimethylstilbestrol was a gift of Dr. Pat Murphy, Eli Lilly and Co.

**ESR Measurements**—ESR measurements were carried out at ambient temperature on solutions contained in a quartz aqueous flat cell, using a Varian E-109 spectrometer operating at 9.5 GHz and employing 100-kHz field modulation. Magnetic field measurements were made with a Radiopan MJ-110 gaussmeter. For microwave frequency measurements an EiP 200 counter was used. Hyperfine splittings were measured (to 0.1 G) either directly from magnetic field separations or from computer simulations of spectra.

**RESULTS**

**Spin Stabilization of \( \alpha \)-Semiquinones**

**Radicals from DES and Analogs**—The addition of tyrosinase to a Tris buffer containing \( \text{Mg}^{2+} \) and DES \( \text{I} \) under aerobic conditions gave a well-resolved ESR spectrum of a semiquinone (Fig. 1A). In the absence of \( \text{Mg}^{2+} \) no ESR spectrum was obtained. The spectrum obtained at a higher modulation amplitude clearly reveals a 1:2:1 feature (Fig. 1B). To further confirm the origin of this coupling we examined a octadeuterated DES, analog 2. The resulting one-line spectrum (Fig. 1C) indicates that the large coupling is due to interaction with the side chain methylene protons. The smallest coupling is assigned to the ortho-proton. The ESR parameters obtained based on computer simulations are shown in Table I.

The ESR spectrum (Fig. 2) obtained during tyrosinase-catalyzed oxidation of dimethylstilbestrol \( \text{I} \) shows evidence for electron delocalization throughout the molecule. Attempts to resolve the hyperfine couplings from protons present in the adjacent aromatic ring were, however, not successful (Fig. 2,

**Scheme 1. Structures of stilbene estrogens examined.**

- 1, DES (\( R=H \)) or 3'-hydroxy-DES (\( R=O\)H); 2, 2,2',3',5,5,5',5''-octadeuteriodiethylstilbestrol (\( R=D \)) or \( 3'-\)hydroxy-2,2',3',5,5,5',5''-heptadeuteriodiethylstilbestrol (\( R=OH \)); 3, dimethylstilbestrol (\( R=H \)) or 3'-hydroxydimethylstilbestrol; 4, E,E-DIES (\( R=H \)) or 3'-hydroxy-(E,E)-DIES; 5, (Z,Z)-DIES (\( R=H \)) or 3'-hydroxy-(Z,Z)-DIES (\( R=OH \)); 6, 2,2',3',5,5,5'',5''-hexadeuterio-(Z,Z)-dienestrol (\( R=OH \)); 7, HEX (\( R=H \)) or 3'-hydroxy-HEX (\( R=OH \)); 8, 3',3'',5,5'',5''-tetradeteriohexestrol (\( R=H \)) or \( 3'-\)hydroxy-3',3'',5,5'',5''-trideuteriohexestrol.

**Fig. 1.** ESR spectrum of the \( \text{Mg}^{2+} \)-complexed \( \alpha \)-semiquinones of catechols of generated \( \textit{in situ} \) during tyrosinase-catalyzed oxidation of DES analogs. A, the incubation contained 2.2 mM DES and 0.35 mg/ml tyrosinase in an aerobic Tris buffer (50 mm, pH 7.5) containing 200 mM \( \text{Mg}^{2+} \). Spectrometer conditions: microwave power, 2 mW; modulation amplitude, 0.1 G; scan time 8 min; time constant, 0.5 s; same as A, but at a modulation amplitude of 1 G; C, same as B, but containing octadeuterated DES.
Mechanism of Radical Production

In tyrosinase-catalyzed reactions the formation of semiquinones usually occurs via a back reaction involving quinones and catechols (25, 26). To test this possible mechanism of formation of semiquinones we investigated radical production in the presence of o-phenylenediamine on the time-dependent ESR of semiquinones formed during tyrosinase-catalyzed oxidation of DES 1 (Fig. 5). o-Phenylenediamine completely quenched the radical production, indicating the o-quinones are precursors of semiquinones in this system (27).

Spin Trapping of Hydroxyl Radicals

Incubations containing DMPO, DES 1, and tyrosinase in phosphate buffer under aerobic conditions yielded a four-line ESR spectrum characteristic of the DMPO-OH adduct (Fig. 6A, top), together with a six-line spectrum attributable to a DMPO-carbon-centered radical adduct. Since the ESR spectrum of the carbon-centered adduct increased in intensity in the presence of added Mg?+-complexed o-semiquinones from stilbene catechol estrogens.

Electron spin resonance data for Mg2+-complexed primary o-semiquinones from stilbene catechol estrogens

<table>
<thead>
<tr>
<th>Parent catechol estrogens</th>
<th>Hyperfine couplings (G)</th>
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<tr>
<td></td>
<td>a1</td>
</tr>
<tr>
<td>1 (R=OH)</td>
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</tr>
<tr>
<td>3 (R=OH)</td>
<td>0.15 (1)</td>
</tr>
<tr>
<td>4 (R=OH)</td>
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<tr>
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</tr>
<tr>
<td>6 (R=OH)</td>
<td>2.7 (1)</td>
</tr>
<tr>
<td>7 (R=OH)</td>
<td>0.38 (1)</td>
</tr>
<tr>
<td>8 (R=OH)</td>
<td>0.38 (1)</td>
</tr>
</tbody>
</table>

* As described under "Materials and Methods," the catechols were generated in situ from the respective estrogens.
* Radical structure contains a double bond between carbon atoms 3 and 4.
* Hyperfine coupling assignments are only tentative.
* Numbers in parentheses indicate the number of equivalent protons.
* Methyl groups are attached to carbon atoms 3 and 4 and the structure also contains a double bond between carbon atoms 3 and 4; a and b refer to carbon atoms 3 and 4.
* Radical structure contains a double bond between carbon atoms 2 and 3 and 4 and 5; couplings from the aromatic protons are not resolved because of high modulation amplitude.
* Radical structure does not contain a double bond in the side chain.
Semiquinones from Stilbene Catechol Estrogens

**FIG. 3.** ESR spectrum of the Mg²⁺-complexed o-semiquinone of catechols generated *in situ* during tyrosinase-catalyzed oxidation of DES analogs. 

**A,** the incubation contained 2.5 mM (E,E)-DIES and 0.5 mg/ml tyrosinase in an aerobic Tris buffer (50 mM, pH 7.5) containing 200 mM Mg²⁺; 

**B,** same as above, but in the presence of (Z,Z)-DIES; 

**C,** same as **B,** but in the presence of the deuterated (Z,Z)-DIES. Spectrometer conditions are the same as Fig. 1A, but at a modulation amplitude of 1 G.

reductant of trace metal ions such as Fe³⁺ is not the superoxide anion. Experiments using o-phenanthroline showed that the actual reductant is the catechol itself (data not shown). Addition of Cu²⁺ to the same incubation mixture (cf. Fig. 6A) greatly enhanced the production of hydroxyl radicals (Fig. 6B, bottom).

**DISCUSSION**

*Semiquinones of DES and Dimethylstilbestrol*—Although free radicals have been detected previously during chemical oxidation of stilbene estrogens (28), none of them have been characterized. This is in part due to their spectral complexity and chemical instability. Spectral parameters from either o-semiquinones or p-semiquinones having a ciefinic double bond have not previously been reported (28). This study illustrates the effect of stereospecific positioning of the olefinic double bond on the electron density distribution in o-semiquinones derived from stilbene catechol estrogens. With DES analogs, the trans-double bond allows for delocalization of the electron over the entire molecule. This is clearly the case for the o-semiquinone derived from 3'-hydroxydimethylstilbestrol as shown below (Scheme 2).

Based on the spectral parameters, the contributions of the different resonance structures shown in Scheme 2 can be assessed. From these it appears that the largest coupling arises from interactions with the methyl groups situated on the β-carbons and that low electron density in the aromatic ring is responsible for the relatively small hyperfine couplings from the aromatic protons. Thus, the aliphatic chain is the most electrophilic site in the molecule and, probably, the preferred site of covalent adduct formation. This analysis is also consistent with the quinone-methide character of o-quinones of the DES analogs. Consistent with this interpretation is the finding that in the reaction of diethylstilbestrol-4',4''-quinone with mercaptoethanol, adduct formation took place at
FIG. 6. The steady-state ESR spectra of DMPO-OH obtained during oxidation of DES by tyrosinase. A top, the incubation contained 2.2 mM DES, 0.3 mg/ml tyrosinase, and 100 mM DMPO in an O2-saturated phosphate buffer (100 mM, pH 7.5). The Me2S0 concentration was 0.85 M, A bottom, same as above, but containing 1.7 M Me2SO; B top, same as A, top, but at a lower spectrometer gain; B bottom, same as above but containing 100 μM Cu++. Symbols denote DMPO-OH and DMPO-carbon-centered adducts, respectively.

Scheme 2. Possible resonance structures of the o-semiquinone derived from 3'-hydroxydimethylstilbestrol.

Semiquinones from Stilbene Catechol Estrogens

Semiquinones of DES and HEX—Electron delocalization over the entire molecule is not possible for the o-semiquinones derived from DES and HEX analogs. Accordingly, the electron density distribution is quite different from that of the DES-0-semiquinone. It is found that the hyperfine couplings of the aromatic ring protons are much higher for the DES- and HEX-o-semiquinones (Table I). Thus the electrophilicities of these semiquinones/quinones should be different from the DES-semiquinone.

It is noteworthy that the ESR parameters of o-semiquinones of (Z,Z)-DES and (E,E)-DES are so different. This can be attributed to the difference in side chain stereoe specificity. The side chain β-proton coupling is critically dependent on the dihedral angle between the C-H bond and the p-orbital containing the unpaired electron and the observed difference in the methyl proton coupling between the o-semiquinone of (E,E) and (Z,Z)-DES can be understood in terms of differing dihedral angles.

Radical-mediated conversions between (Z)- and (E)-DES as well as between DES and DIES have been demonstrated in peroxidatic systems (33, 34). The DES-p-semiquinone is the suggested intermediate in the isomerization between (Z)-DES and (E)-DES (8), whereas both p-DES-semiquinone and -quinone are the proposed intermediates in the conversion of DES to DIES (8, 9). Based on this, (Z)-DES has been used as a marker product for the one-electron reduction of DES-p-quinone (8). In an earlier publication (35), metabolic conversion of 3'-OH-DES to 3'-OH-DIES via a reactive intermediate has been postulated. Since we did not observe the ESR spectrum of DIES-o-semiquinone during tyrosinase-catalyzed oxidation of DES, it is reasonable to conclude that DES-o-semiquinone is not involved.

Several investigators have invoked the presence of either the o- or p-semiquinone during metabolism of stilbene estrogens in the target organ of carcinogenesis (1–3). Most p-semiquinones and some hydroxy-substituted o-semiquinones undergo redox-cycling in the presence of molecular oxygen forming superoxide and the corresponding quinone (36). For example, the DES-p-semiquinone has been shown to undergo air-oxidation forming the DES-p-quinone and superoxide during micromosomal oxidation/reduction of DES and DES-p-quinone (9). With DIES and HEX analogs, the oxygen activation via similar radical species is not possible, since these compounds lack the conjugated double bond system. Thus, it appears that a pathway (common to all stilbene estrogens) that is capable of oxygen-activation is through formation of stilbene catechol estrogens. Almost all stilbene catechol estrogens have been found to catalyze the formation of hydroxyl radical in the presence of the Fe++-EDTA complex (data not shown). The reduction of Fe++-EDTA by these catechols presumably occurs by an outer-sphere electron transfer (37). It is notable that DES-catechol appears to favor the reductive decomposition of the iron complex despite the fact that it has an electron withdrawing olefinic

the quinone methide double bond (29). On the other hand, the p-benzo- and -naphthoquinones, which lack quinone methide character, form sulphydryl adducts at the aromatic centers (30, 31).

Previously, it has been shown, by NMR spectroscopy, that the DES-p-quinone is planar (32). The ESR parameters of dimethylstilbestrol-o-semiquinone suggest that there is extensive electron delocalization (Scheme 2). Although we have not been able to resolve the small aromatic couplings from the distant benzene ring, it is likely that these o-semiquinones are also planar given the structural similarity between DES-p-quinone and DES-o-quinone.

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bond; most catechols with electron-withdrawing groups tend to form a bidentate complex without effecting reduction (38). The DES-catechol, in the presence of the Fe$^{3+}$-EDTA complex, has been shown to cause extensive cellular damage, including DNA strand breaks (39). Based on the present results, the hydroxyl radicals are most likely to be involved in the DNA damage.

Catechol-Fe$^{3+}$ complexes have been shown to be only weakly redox-active (40). However, in the present study hydroxyl radicals were detected even in the presence of added chelators such as EDTA which enhance redox activity. Therefore, it appears that stillbene catechol-Fe$^{3+}$ complexes are able to catalyze the Fenton reaction. The enhanced production of hydroxyl radicals in the presence of micromolar concentrations of Cu$^{2+}$ is noteworthy. It was recently shown that some plant-derived catechols cause DNA strand scission in a reaction catalyzed by Cu$^{2+}$ and O$_2$ (41). These catechols or their copper complexes presumably associate with DNA prior to the production of oxyradicals (41). The Cu$^{2+}$-dependent sequence-selective DNA strand scission reactions are now an active area of research (42, 43).

In summary, we have characterized the o-semiquinones from stillbene catechol estrogens and based on the ESR data, we conclude that the electrophilicities of these stillbene o-quinones are quite different. We have also proposed in this paper a new mechanism of hydroxyl radical production from stillbene catechol estrogens.

**REFERENCES**