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XANTHINE OXIDASE-CATALYZED REDUCTION OF ESTROGEN QUINONES TO SEMIQUINONES AND HYDROQUINONES

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Abstract—Metabolic redox cycling between the stilbene estrogen diethylstilbestrol (DES) and diethylstilbestrol-4′,4″-quinone (DES Q) has been demonstrated previously. The xanthine and xanthine oxidase-catalyzed reduction of estrogen quinone has been studied in this work to understand the role of metabolic redox cycling in estrogen metabolism. Xanthine and xanthine oxidase catalyzed the reduction of DES Q to 44% *Z*-DES and 9% *E*-DES. This reaction was inhibited by the addition of superoxide dismutase or by a lack of oxygen (under anaerobic conditions). DES Q was also reduced in a non-enzymatic reaction by superoxide radicals generated by potassium superoxide and crown ether. The reaction between the $O_2^{\cdot}$ and DES Q was also investigated by an electron spin resonance spin-trapping technique. The superoxide anion generated in an oxygen-saturated xanthine and xanthine oxidase system was detected as 5,5-dimethyl-1-pyrroline-1-oxide-superoxide adduct. The addition of DES Q or 2,3-estradiol quinone totally inhibited the formation of this adduct. The reduction of DES Q by superoxide radicals was taken as evidence that this reaction was one possible mechanism of xanthine and xanthine oxidase-mediated reduction. In addition, reduction of DES Q by direct electron transfer to quinone by the enzyme may also occur. The intermediate formation of semiquinone free radicals in the reduction is implied by the nature of the single electron transfer reactions and, in addition, has been demonstrated for the catechol estrogen by electron spin resonance measurements. It is concluded that the reduction of estrogen quinones to their hydroquinones by xanthine oxidase occurs by both one electron transfer to the quinone and by formation of superoxide which then reduces the quinone.

Estrogens, such as diethylstilbestrol (DES)§ or the catechol estrogen 2-hydroxyestradiol (2-OH-E_2), may undergo metabolic redox cycling mediated by several enzyme systems (reviewed by Liehr and Roy [1]). For instance, cytochrome P450IA1 [2] prostaglandin synthase [3], or various peroxidases [4, 5] oxidize DES or catechol estrogens to corresponding quinones (Fig. 1). The reduction of these quinones to their hydroquinones is catalyzed by cytochrome P450 reductase, cytochrome b_5 reductase in the presence or absence of cytochrome b_5, or by quinone reductase [2, 6, 7]. The reduction of diethylstilbestrol-4′,4″-quinone (DES Q) by NADPH-dependent cytochrome P450 reductase has been shown to be accompanied by the generation of superoxide radicals [7]. This formation of $O_2^{\cdot}$ has been taken as evidence that DES semiquinone is an intermediate in the NADPH-dependent cytochrome P450 reductase-catalyzed reduction. Semiquinone free radicals are known to react with molecular oxygen to produce $O_2^{\cdot}$ and the parent quinone [8]. The reverse reaction, the reduction of DES Q, has been reported to be mediated by xanthine and xanthine oxidase, a system known to generate superoxide radicals [9]. In this reaction, DES semiquinone formation was assumed because of a concomitant reduction of cytochrome *c*. Direct evidence of semiquinone formation or of the disappearance of DES Q substrate in the reaction with xanthine and xanthine oxidase has not been obtained. Moreover, the mechanism of this reaction has also remained unclear. It is possible that DES Q had been reduced by superoxide, the product of the xanthine and xanthine oxidase reaction, or directly by the enzyme. This study has been carried out to generate direct evidence for the xanthine and xanthine oxidase-catalyzed reduction of estrogen quinones to semiquinones and hydroquinones by product analysis and electron spin resonance (ESR) measurements and to distinguish between the two mechanistic possibilities mentioned above.

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§ Abbreviations: DES, diethylstilbestrol; *E*-DES or *Z*-DES, *E*- or *Z*-isomers or *trans*- or *cis*-isomers of diethylstilbestrol; DES Q, diethylstilbestrol-4′,4″-quinone; DIES, *Z*,*Z*-dienestrol; 2-OH-E_2, 2-hydroxyestradiol; ESR, electron spin resonance, DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; and DMPO-OOH, DMPO-superoxide adduct.

MATERIALS AND METHODS

Chemicals. *E*-DES, xanthine and xanthine oxidase were purchased from the Sigma Chemical Co., St. Louis, MO. 18-Crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), and potassium superoxide were obtained from the Aldrich Chemical Co., Milwaukee, WI. *Z*-DES was a gift of Dr. P. Murphy, Eli Lilly & Co., Indianapolis, IN. Desferal mesylate was a gift of the Ciba-Geigy Co., Summit, NJ. DES Q and *Z*,*Z*-dienestrol (DIES) were prepared as described

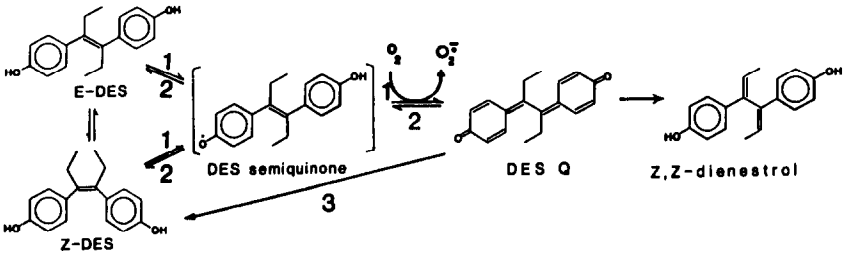

Fig. 1. Metabolic redox cycling between DES and its corresponding quinone DES Q. Oxidations are catalyzed by cytochrome P450 (1) and reductions of DES Q by cytochrome P450 reductase (2) [6]. DES Q may spontaneously rearrange to *Z,Z*-dienestrol, the marker product of oxidation. The preferred product of reduction of DES Q is *Z*-DES and thus it serves as a marker of reduction. The semiquinone has been shown previously to react with molecular oxygen to form superoxide radicals [7]. Quinone reductase (3) reduces DES Q directly to *Z*-DES in a two electron transfer bypassing the formation of semiquinone intermediates.

previously [10]. The purity of DES Q was determined by ultraviolet and nuclear magnetic resonance spectroscopy, high pressure liquid chromatography and, after rearrangement to DIES, by gas chromatography–mass spectrometry. DMPO was purified by vacuum distillation. All solvents and common chemicals used were either analytical grade or of the highest grade available.

Instrumentation. UV spectra were recorded on a Hewlett–Packard, model 8452A, diode array spectrophotometer. High pressure liquid chromatography analyses were carried out using a Waters Associates (Milford, MA) instrument consisting of two solvent delivery systems, model 510 and model 501, an automated gradient controller, and a model 490 multiwavelength detector. Data were recorded by a Waters model 740 data module. ESR measurements were carried out using a Varian E-109 spectrometer, Radiopan MJ-110 gaussmeter and EiP 200 Counter.

Xanthine oxidase system. The reaction mixture consisted of 50 μM xanthine, 0.005 unit/mL xanthine oxidase, 10 mM phosphate buffer, pH 7.5, and 30 μM DES Q. Reactions were carried out at room temperature.

Generation of superoxide radicals by potassium superoxide and crown ether. The reaction mixture consisted of 3 mmol potassium superoxide, 0.3 mmol 18-Crown-6 and 100 μM DES Q in acetonitrile solution [11]. Reactions were carried out at room temperature for 60 min.

UV monitoring of the disappearance of DES Q. The reduction of DES Q was monitored as a gradual decrease in UV absorption in the range of 200–650 nm recorded every 30 sec for 120 sec. Changes in absorbance at 312 and 340 nm were used to calculate the rate of reduction of DES Q.

Analyses of products of the reduction of DES Q. The reduction of DES Q to *Z*-DES, a marker product of reduction [7], was analyzed by a high pressure liquid chromatography method. Products were extracted from the reaction mixture and analyzed by high pressure liquid chromatography as described previously [7]. The formation of the reduction product *Z*-DES was expressed as the percent of total stilbenes extracted.

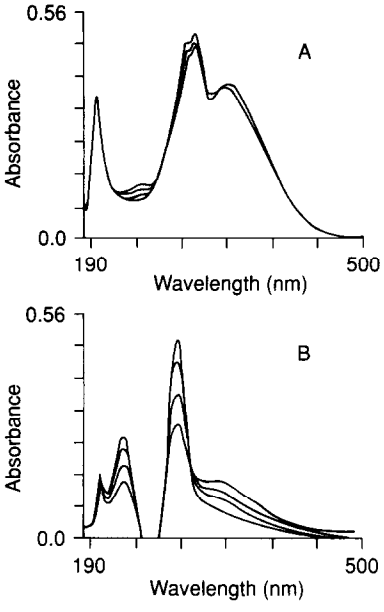

Fig. 2. Reduction of DES Q by xanthine and xanthine oxidase as monitored by UV spectroscopy (panel B). Control incubations were carried out in the absence of enzyme (panel A). UV spectra were recorded 0, 30, 60, and 90 sec after addition of DES Q. Decreases in absorption at 312 nm, the absorption maximum of DES Q [10], were used to calculate the rate of reduction of DES Q. A concomitant increase in absorption was observed at 242 nm, which is characteristic of the reduction product DES.

ESR measurements. ESR measurements were carried out at ambient temperature. The solutions were contained in a quartz aqueous flat cell, using an ESR spectrometer operating at 9.5 GHz and employing 100 kHz field modulations.

RESULTS

Reduction of DES Q. DES Q was reduced rapidly to DES in the presence of xanthine and xanthine oxidase. The reduction of DES Q was monitored

Table 1. Reduction of DES Q by xanthine and xanthine oxidase or by potassium superoxide

Expt. No.	Reaction conditions	% of total stilbenes extracted *E*-DES	*Z*-DES	DIES
Xanthine and xanthine oxidase-mediated reduction				
I	DES Q, xanthine	<0.1	<0.1	99.8
II	DES Q, xanthine oxidase	<0.1	<0.1	99.8
III	DES Q	<0.1	<0.1	99.9
IV	*E*-DES, xanthine, xanthine oxidase	99.9	<0.1	<0.1
V	DES Q, xanthine, xanthine oxidase	9.2	44.0	46.8
VI	DES Q, xanthine, xanthine oxidase, desferal mesylate	10.8	43.0	46.2
VII	DES Q, xanthine, xanthine oxidase, and superoxide dismutase	6.8	29.5‡	63.7
VIII	DES Q, xanthine, xanthine oxidase, and boiled superoxide dismutase	10.5	46.0	45.5
IX	DES Q, xanthine, xanthine oxidase, and bovine serum albumin	9.8	48.0	42.2
X	DES Q, xanthine, xanthine oxidase (anaerobic)*	8.6	28.5‡	62.9
XI	DES Q, xanthine, xanthine oxidase (anaerobic)†	5.8	14.6§	79.6
Potassium superoxide-mediated reduction				
XII	DES Q, potassium superoxide, and 18-Crown-6	14.5	65.5	20.0
XIII	DES Q, 18-Crown-6	<0.1	<0.1	99.8

Mixtures of 5.0×10^{-3} units of xanthine oxidase, 0.05 mM xanthine, and 30 μM DES Q or *E*-DES were incubated for 6 min at 30°. Reactions in the presence of superoxide dismutase were carried out with 1500 units of the enzyme (200 μg) added prior to mixing with the substrate. Control reactions were carried out in the presence of 100 μM desferal mesylate or boiled superoxide dismutase (100° for 5 min) or bovine serum albumin (200 μg). The non-enzymatic generation of superoxide radicals was achieved by mixing 3 mM potassium superoxide and 0.3 mM 18-Crown-6 in acetonitrile prior to addition of 100 μM DES Q. The reduction of DES Q in all experiments was analyzed by UV spectral changes and by high pressure liquid chromatography analysis of the products. Values are means of 3–4 experiments and are expressed as percent of total stilbenes extracted. Statistical calculations were done with absolute values.

* Incubations under anaerobic conditions were done in the same way as reactions under air except that N_2 gas was bubbled through the incubation mixture for 5 min before starting the reaction.

† Incubations under anaerobic conditions were done in the same way as reactions under air except that N_2 gas was bubbled into all solvents for 5 min. The reactions were then carried out in a hood in a stream of N_2.

‡ $P < 0.03$ compared to Expt. V.

§ $P < 0.01$ compared to Expt. V.

spectrophotometrically by analyzing the gradual disappearance of UV absorptions of DES Q at 312 and 340 nm (Fig. 2). In the presence of xanthine and xanthine oxidase, DES Q was reduced to DES at a rate of approximately 2 nmol/min. After 120 sec, the substrate concentration was decreased by 38.6% (Fig. 2B). In control incubations in the absence of xanthine or xanthine oxidase, only about 1% of DES Q had reacted in 120 sec.

The reduction of DES Q to DES by xanthine and xanthine oxidase was further confirmed by analysis of the reaction products using high pressure liquid chromatography. It was found that 44% of DES Q had been converted to *Z*-DES, a reduction marker product [7], (Table 1, Expt. V). *E*-DES (9.2%) may also be a product of this reduction of DES Q or may have been formed by equilibration between the *Z*- and *E*-forms of the stilbene estrogen as described previously [12]. The remaining DES Q (46.8%) had spontaneously rearranged to DIES as described previously [10]. *E*-DES was not a substrate for xanthine oxidase and remained unchanged (Table 1, Expt. IV). Preincubating the mixture with iron chelating agents such as 100 μM desferal mesylate (Table 1, Expt. VI) or EDTA (data not shown) before starting the reaction did not influence the xanthine and xanthine oxidase-catalyzed reduction of DES Q to *Z*-DES. The formation of *E*- and *Z*-DES from DES Q by xanthine and xanthine oxidase under anaerobic conditions was inhibited (Table 1, Expt. X and XI) compared to values obtained in the presence of air.

The addition of superoxide dismutase (1500 units) inhibited the xanthine and xanthine oxidase-catalyzed reduction of DES Q to DES by 33%. This inhibition indicates that superoxide radical, generated by the xanthine and xanthine oxidase system, may be the reducing agent of DES Q, because there was no such inhibition in the presence of inactive superoxide dismutase (boiled at 100° for 5 min) or equimolar concentrations of bovine serum albumin (200 μg). The direct role of superoxide radical in the reduction of DES Q to DES was further illustrated by the reduction of DES Q in an enzyme-free reaction known to generate $O_2^{\cdot}$ (Table 1, Expt. XII). Superoxide radicals generated by potassium superoxide and crown ether reduced DES Q to *Z*-DES and a small amount of *E*-DES in an 80% yield.

In summary, the data show that DES Q is reduced to *Z*-DES and some *E*-isomer either by a xanthine and xanthine oxidase-mediated reaction or by potassium superoxide and crown ether.

ESR measurements. The reaction between the

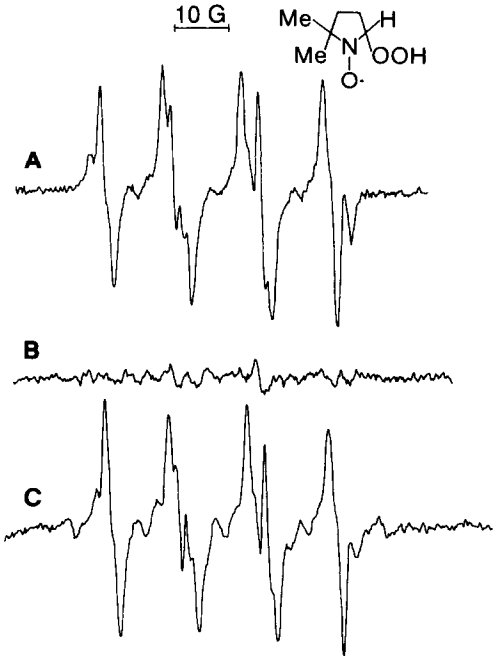

Fig. 3. Effects of DES Q and DES on the concentration of the DMPO-OOH adduct formed during the xanthine oxidase-catalyzed oxidation of xanthine in phosphate buffer, pH 7.4, saturated with oxygen. (A) ESR spectrum before addition of stilbene; (B) in the presence of 30 μM DES Q; and (C) in the presence of 30 μM DES.

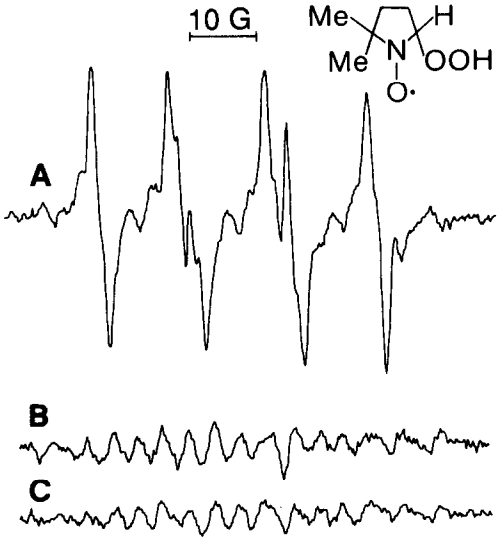

Fig. 4. Effects of 2-OH-2-E_2 and 2,3-estradiol quinone on the concentration of the DMPO-OOH adduct formed during the xanthine oxidase-catalyzed oxidation of xanthine in phosphate buffer, pH 7.4, saturated with oxygen. (A) ESR spectrum before addition of steroid; (B) in the presence of 30 μM 2-OH-E_2; and (C) in the presence of 30 μM 2,3-estradiol quinone.

superoxide anion and DES Q was investigated also by an ESR-spin trapping technique. The superoxide anion generated in an oxygen-saturated xanthine and xanthine oxidase system was detected as DMPO-superoxide adduct (DMPO-OOH) (Fig. 3A). The addition of DES Q totally inhibited the formation of DMPO-OOH (Fig. 3B). This quenching effect was observed only with DES Q, but not with DES (Fig. 3C). This implies that the superoxide radical reacts with DES Q but not with DES.

Since DES semiquinone was suspected to be one of the intermediates of this reaction, we attempted to detect DES semiquinone by direct ESR measurements. At physiological pH, we were unable to detect any spectral evidence of DES semiquinone. However, at higher pH (pH 9.5), a five-line ESR spectrum was obtained during reduction of DES Q by xanthine and xanthine oxidase. The spectral parameters of the five-line spectrum were identical to those of *p*-benzosemiquinone. It is likely that the DES semiquinone is unstable and had decomposed to *p*-benzosemiquinone at higher pH.

In contrast to the lack of reaction between $O_2^{\cdot}$ and DES, both 2-OH-E_2 and 2,3-estradiol quinone were found to totally inhibit the formation of DMPO-OOH (Fig. 4B and C). This suggests that the superoxide anion reacts with both the 2-OH-E_2 and 2,3-estradiol quinone. In both reactions, the expected intermediate was the 2-OH-E_2 semiquinone which was detected by ESR-spin stabilization (data not shown) [13].

DISCUSSION

The ESR data demonstrate that the xanthine oxidase-mediated superoxide radical formation was inhibited by estrogen quinones. The reduction of estrogen quinones to their respective hydroquinones, *Z*-DES and 2-OH-E_2, during this reaction was demonstrated by UV monitoring and by product analysis and may be responsible for this inhibition. The reduction likely proceeds by single electron transfer according to Scheme 1 below.

$$\text{DES Q} + O_2^{\cdot} \rightleftharpoons \text{DES semiquinone} + O_2 + H^+$$

$$\text{DES semiquinone} + O_2^{\cdot} \rightleftharpoons \text{Z-DES} + O_2 + H^+$$

$$\text{2,3-Estradiol quinone} + O_2^{\cdot} \rightleftharpoons \text{2-OH-}E_2\text{ semiquinone} + O_2 + H^+$$

$$\text{2-OH-}E_2\text{ semiquinone} + O_2^{\cdot} \rightleftharpoons \text{2-OH-}E_2 + O_2 + H^+$$

Scheme 1

The intermediate formation of semiquinone free radicals is implied by the nature of the single electron transfer reactions and, in addition, has been demonstrated for the catechol estrogen by ESR measurements [13]. The equilibrium constant for the initial reaction, reduction of quinone to semiquinone, shown in Scheme 1 must be close to 1, because this reaction resembles closely that of $O_2^{\cdot}$ and *p*-benzoquinone, which has an equilibrium constant close to unity [14]. High concentrations of superoxide radicals and/or estrogen quinones stimulate the reduction and result in formation of the semiquinones

and hydroquinones. On the other hand, elevated concentrations of DES semiquinone, generated by cytochrome P450 reductase-dependent reduction of DES Q, in the presence of molecular oxygen result in formation of superoxide radicals [7].

The reduction of estrogen quinones by superoxide radicals points to this reaction as one possible mechanism of xanthine and xanthine oxidase-mediated reduction. The superoxide generated by the enzyme-catalyzed reaction subsequently reduces the estrogen quinones. The inhibition of the reduction under anaerobic conditions or in the presence of superoxide dismutase supports the proposed mechanism of xanthine and xanthine oxidase-mediated reduction via generation of $O_2^{\cdot}$. In addition, direct electron transfer to benzoquinone by the enzyme has been demonstrated previously [15] and may also operate in the reduction of estrogen quinones. The extent of reduction of estrogen quinones to their hydroquinones either by direct electron transfer from the enzyme to the substrate or by superoxide radical remains to be ascertained.

The biological significance of the oxidation/reduction reactions shown above may lie in the possible interconversion of free radicals. The formation of semiquinone free radicals via reduction of quinones by superoxide indicates a possible escape from cellular detoxification mechanisms for superoxide. If these semiquinone free radicals react with and damage cellular macromolecules, the depletion of semiquinones may be the driving force for additional reduction of quinones by superoxide. Thus, the biological result of the equilibrium between quinone/superoxide and semiquinone/oxygen may be enhanced toxicity by semiquinone free radicals generated within the cell.

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