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Comparison of estrogen-derived *ortho*-quinone and *para*-quinol concerning induction of oxidative stress

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Abstract

Ortho-quinones formed from catechol estrogens are considered prooxidants due to the production of superoxide radical anions through redox cycling via semiquinones. *Para*-quinols have been identified as novel metabolites of and as the major products of hydroxyl-radical scavenging by estrogens. Cycling of these compounds has also been discovered, because they are converted back to the parent estrogen via reductive aromatization in vitro and in vivo. We hypothesized that, unlike *ortho*-quinones, *para*-quinols do not induce oxidative stress due to this cycling. Like the estrogen itself, the 17 β -estradiol-derived *para*-quinol (10 β ,17 β -dihydroxyestra-1,4-diene-3-one) did not induce oxidative stress, as the rate of hydrogen peroxide production during the incubations of the compounds in various tissue homogenates was not significantly different from that of the control experiments performed without the addition of a test compound. We also confirmed that the estrogen metabolite estra-1,5(10)-dien-3,4,17-trione (estrone 3,4-quinone) was a profound prooxidant due to redox cycling, especially in uterine tissue. Therefore, we concluded that *para*-quinols do not induce oxidative stress.

1. Introduction

The antioxidant activity of estrogen has been recognized recently, particularly in neuroprotection exerted by this gonadal steroid [1,2]. However, the capacity of the estrogens to reduce the oxidative stress has been a topic of controversy, since they could be converted into prooxidant and carcinogen metabolites. In particular, catechol estrogens that are produced by cytochrome P450-mediated hydroxylation of the estrogens [3] in the position adjacent to the phenolic hydroxyl group and further oxidized to *ortho*-quinones have been implicated in these detrimental effects. From these *ortho*-quinones, superoxide radical anion (O₂^{•-}) is generated from molecular oxygen via semiquinone formation in a perpetual cyclic mechanism known as redox cycling, as exemplified in Fig. 1 [4]. Therefore, this redox cycling induces oxidative stress that has been linked to estrogen-related carcinogenicity [5,6]. The major phase

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A metabolic pathway of estrogens involving 2-hydroxylation and implicating 2,3-quinones into redox cycling is regarded as benign, because it has not been linked to mutagenesis in cell culture studies or to carcinogenesis in animal models [7]. On the other hand, estrogen-3,4-quinones were identified as endogenous tumor initiators [8].

The metabolic formation of *para*-quinols from estrone (E1) and 17 β -estradiol (E2) is catalyzed by various CYP isozymes [9], and a non-metabolic pathway involving the capture of hydroxyl (HO $\cdot$) radicals has been recognized recently [10]. From these *para*-quinols, the phenolic parent compounds can be regenerated by reductive aromatization with NAD(P)H as a cellular reductant [10,11]. This quinol-based cycle shown in Fig. 2 has been implicated in the antioxidant neuroprotective action of estrogens [10].

We have hypothesized that the prooxidant effect of the *ortho*-quinones is not manifested by *para*-quinols, because molecular oxygen is not involved in the quinol-based cycle and their chemical structures exclude the formation of radical intermediates. In order to prove this hypothesis, we incubated different rat tissue homogenates (brain, liver, uterus and retina) with *para*-quinol obtained from E2, and measured hydrogen peroxide (H₂O₂) production compared to untreated control as an indicator of excess O₂ $\cdot^-$ formation. In addition to E2, H₂O₂ production induced by estradiol-1,5(10)-diene-3,4,17-trione (3,4-E1Q, an estrogen-derived *ortho*-quinone well-known as a potent prooxidant [12] and an endogenous tumor initiator [8]) was measured as a reference. We have also chosen 3,4-E1Q as a positive control in our experiments, because the isomeric estrogen-2,3-quinones are short-lived, which apparently reduces their prooxidant effects compared to the considerably longer-lived estrogen-3,4-quinones [7].

We focused our studies on the liver as the center of the body's metabolic activity [13], on the brain and retina in which oxidative stress has been associated with various degenerative diseases and aging [14–16], and on the uterus that, besides being the target organ for estrogenic endocrine effect [17], has been studied regarding estrogen-related oxidative stress [18].

2. Materials and Methods

3,17 β -Dihydroxyestra-1,3,5(10)-trien (17 β -estradiol, E2) and 3,4-dihydroxyestra-1,3,5(10)-trien-17-one (4-hydroxyestrone, 4-OH-E1) were obtained from Steraloids (Newport, RI) and were used without further purification based on the report of purity supplied by the manufacturer. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA). Tissues were obtained from two to six ovariectomized Sprague-Dawley rats (230–250 g body weight, about 4 months old, three weeks after the removal of the ovaries) supplied by Charles River Breeding Laboratories (Wilmington, MA).

Oxidation of 4-OH-E1 to 3,4-E1Q was performed using activated manganese dioxide, as described by Abul-Hajj [19]. We confirmed by high-performance liquid chromatography (HPLC) that the crude 3,4-E1Q was of >90% purity; hence, it was used without purification because of its limited stability [19]. 10 β -Hydroxyestra-1,4-diene-3-one (E2-quinol) was prepared by the oxidation of E2 with 3-chloroperoxybenzoic acid using benzoyl peroxide as a radical initiator [20]. E2-quinol was meticulously purified by preparative thin-layer chromatography (TLC) to obtain it with >99% purity by HPLC.

H₂O₂ production in tissues was determined using the method described Nutter *et al.* [12] with some modifications [10]. Briefly, brain, liver, retina and uterus homogenates (0.2% w/v in 0.1M PBS, pH 7.4, final volume of 100 μ L) were incubated without and with E2 or the estrogen derivatives (100 μ M), NADPH (200 μ M) and sodium azide (20 μ M) at 37°C for 30 minutes in a shaking water bath. Then, 2 mL of color-developing solution (100 μ M xylenol orange, 250 μ M ferrous ammonium sulfate, and 100 μ M sorbitol in 25 mM sulfuric acid) was added,

and the mixture was allowed to stand at room temperature for 45 min before spectrophotometric analysis. Absorbance was recorded at 560 nm on a Beckman DU 640 (Fullerton, CA) instrument. Quantification of H₂O₂ was done by calibrating with serially diluted H₂O₂ standard solutions. The assays were performed in triplicate. The protein content of tissues was determined spectrophotometrically by the Bradford dye-binding method [21] using bovine serum albumin as a reference.

Rates of H₂O₂ production were expressed in units of nmol·h⁻¹·mg protein⁻¹ (mean ± standard deviation). One-way analysis of variance (ANOVA) followed by post hoc Dunnett's test was done to test for statistically significant differences ($P < 0.05$) compared to control (tissue incubation without added compound).

Formation of E2 from E2-quinol during incubation was confirmed by liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS). The assays were run in triplicate. Solution samples (0.5 mL solution) removed after 2.5 and 30 min, respectively, were mixed with aliquot of aqueous 1-μM d₃-E2 (Aldrich, Milwaukee, MN, USA). Ethyl acetate (3 × 0.5 mL) was added, the mixture was vortexed for 1 min and centrifuged at 10,000 rpm for 5 min. The organic layer was removed and the solvent was evaporated at room temperature under nitrogen stream. The sample residues were dissolved in 100 μL of mobile phase, respectively, and 20 μL of the solution was injected for analysis. LC separation was done using a Supelco (Bellefonte, PA, USA) 5 cm × 2.1 mm i.d. Discovery HS C-18 reversed-phase column with 0.3 mL/min water:acetonitrile:acetic acid (64:35:1, v/v) as a mobile phase. Mass spectra were recorded on a quadrupole ion-trap instrument (LCQ; Thermo Electron, San Jose, CA, USA) using positive-ion atmospheric-pressure chemical ionization (APCI) [10,11]. Identification of E2 was based on retention time (t_R) and MS/MS of authentic reference compound (Steraloids). MS/MS product-ion scans were obtained after collision-induced dissociation with helium as the target gas. For quantitative analyses, the sum of the three most intense product ions was used to extract chromatograms from MS/MS scans. E2 concentrations (in μM) were obtained by taking the ratio of the analyte to internal standard (d₃-E2) peak areas of the extracted chromatograms.

3. Results

To avoid interference from the endogenous hormone, we conducted the incubations in tissues obtained from ovariectomized rats. H₂O₂ production was determined in brain, liver, uterus and retina homogenates in the presence of E2, E2-quinol, and 3,4-E1Q. Control samples, with no estrogens or estrogen derivative added, were also measured and used in statistical evaluations. As summarized in Table 1, the rate of H₂O₂ production increased substantially in the presence of 3,4-E1Q, especially in the uterus homogenate. No statistically significant change was detected in the presence of E2 and E2-quinol. A slight increase compared to control in the H₂O₂ formation was obtained in the uterus homogenate upon addition of E2-quinol. However, an attenuation of H₂O₂ formation was consistently observed in the presence of the E2-quinol in the brain, retina and liver, and E2-quinol steadily outperformed E2 in all tissues examined.

LC/MS/MS assay shown in Fig. 3 has unequivocally indicated that E2-quinol converts to E2 in the incubation medium (containing 200 μM of the coenzyme NADPH [10,11]) under the conditions of our experiments. Initial rate of E2 formation from E2-quinol (100 μM) was approximately 33 ± 3 nM·min⁻¹ based on quantitative analysis of samples removed after a short (2.5-min) incubation time.

4. Discussion

Estrogen metabolism has received attention due to the recognition that oxidative metabolism of the hormone could play a very important role in the initiation and evolution of estrogen-related cancerous processes. E2 is transformed in cells to multiple hydroxylated metabolites with various cytochrome P450 (CYP) complexes; among others to the catechol estrogens 2- and 4-hydroxyestrones (2- and 4-OH-E1). Generally, 2-hydroxylation is regarded as a benign metabolic pathway because it has not been linked to mutagenesis in cell culture studies or to carcinogenesis in animal models, whereas 4-OH-E1 was found to be carcinogenic in male Syrian golden hamster kidney tumor model [22,23] and in the uterus of CD-1 mice [24]. Catechol estrogens can easily be oxidized (often by autooxidation) to the corresponding *ortho*-quinone derivatives (Fig. 1) that have the potential to both initiate and promote deleterious processes by generating reactive oxygen species (ROS), which leads to oxidative stress and thus, cellular damage [7,26,27]. The *ortho*-quinone *estra-4,10(1)-diene-2,3,17-trione* (2,3-E1Q) formed from 3,4-dihydroxyestra-1,3,5(10)-trien-17-one (2-OH-E1) has considerably shorter biological half-life than 3,4-E1Q (47 s *versus* 12 min [7]), which may also explain the 'benign' nature of 2,3-E1Q in comparison to 3,4-E1Q [8]. *Ortho*-quinones are electrophilic compounds and act as Michael acceptors [28] that are able to alkylate nucleophilic sites on peptides, proteins and/or nucleic acids and, therefore, can form covalent adducts with DNA. While this 2,3-E1Q forms stable DNA adducts, 3,4-E1Q reacts with guanine (G) or adenine (A) resulting in the destabilization of the glycosyl bond and, thus, removal of these bases from DNA [28–30]. Therefore, we selected 3,4-E1Q as a positive control (prooxidant) in our studies. Indeed, this estrogen-derived *ortho*-quinone has significantly increased H₂O₂ production in all studied rat tissues. The origin of this excess H₂O₂ in tissue is principally O₂^{•−} (see Fig. 1) and involves the enzyme superoxide dismutase (SOD) [4,6,12]. The prooxidant effect of 3,4-E1Q was especially profound in the uterus homogenate of ovariectomized animals.

The antioxidant activity of E2 has been recognized and the hormone has been considered among the agents that potentially prevent the brain from oxidative stress-related disorders such as Alzheimer's disease [31] and stroke [32–34]. The neuroprotective effects of estrogens has been demonstrated *in vivo* and *in vitro* under a wide range of toxic conditions including models of acute neuronal stress, as well as neurodegeneration [35]. A phenolic A-ring in the steroids has also been considered a requirement for estrogen neuroprotection [36,37]. Our earlier studies have identified *para*-quinol as the key intermediate in a previously unrecognized antioxidant cycle for estrogen during radical scavenging [10,11]. *Ortho*-quinones and *para*-quinols may be considered structurally related compounds (Fig. 1 and 2). This raises the question whether the *para*-quinol cycle, like the redox cycling from *ortho*-quinols, also induces oxidative stress? However, we have clearly shown that E2-quinol does not act as a prooxidant, as the rate of H₂O₂ production in the presence of this compound is not significantly different from that of the corresponding control experiments for all the tissues studied (Table 1). As we have demonstrated earlier [10,11] and by the experiments summarized here in Fig. 3, estrogen-derived *para*-quinols can convert back to estrogens that possess antioxidant activity. Therefore, E2-quinol may even reduce oxidative stress through the production of E2.

In conclusion, our studies have indicated that oxidative stress-related properties of *para*-quinols are different from those of the *ortho*-quinones. Specifically, prooxidant effects of the *ortho*-quinones are not manifested by the estrogen-derived *para*-quinols, because molecular oxygen and/or free-radical intermediates are not involved in the quinol-based cycle. Consequently, *para*-quinols may be used as prodrugs for neuroprotectant estrogens.

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Fig. 1.

Formation and redox cycling of estradiol-4,10(1)-dien-2,3,17-trione (2,3-E1Q) and estradiol-1,5(10)-dien-3,4,17-trione (3,4-E1Q) via semiquinones [3,4]. (CYP, cytochrome P450; 17β-HSD, 17β-hydroxysteroid dehydrogenase.)

Fig. 2.
Cycling of 3,17β-dihydroxyestra-1,3,5(10)-triene (estradiol) via the *para*-quinol 10β,17β-dihydroxyestra-1,4-diene-3-one.

Fig. 3.

Reductive aromatization of E2-quinol to E2 (LC/APCI-MS/MS analysis):

A) Chromatographic traces are the selected reaction monitoring (SRM) of m/z 271 $[M+H-H_2O]^+ \rightarrow m/z$ 161, 175 and 189 for E2-quinol and the SRM of m/z 255 $[M+H-H_2O]^+ \rightarrow m/z$ 133, 159, 173 for E2. **B)** The peak at $t_R = 3.5$ min is unequivocally identified, based on coelution with an authentic reference compound and identical MS/MS (shown in the chart together with the probable origin of the major fragments observed), as E2.

Table 1

Rates of H₂O₂ production (nmol·h⁻¹·mg protein⁻¹, mean ± standard deviation) at 37°C in selected tissue homogenates (0.2%, w/v, pH 7.4) of ovariectomized rats in the absence and presence of E2, 3,4-E1Q and E2-quinol (100 µM, respectively). Asterisks indicate statistically significant differences (ANOVA followed by *post hoc* Dunnett's test, *P*<0.05) from the respective controls.

Compound	Tissue			
	Liver	Brain	Retina	Uterus
None (control)	58 ± 16	155 ± 18	178 ± 3	173 ± 12
E2	45 ± 17	170 ± 5	177 ± 20	300 ± 20
3,4-E1Q	687 ± 40*	1050 ± 8*	580 ± 5*	6646 ± 186*
E2-quinol	34 ± 18	125 ± 19	163 ± 9	266 ± 31