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Short Communication

QUINOL-BASED METABOLIC CYCLE FOR ESTROGENS IN RAT LIVER MICROSOMES

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ABSTRACT:

According to a recently reported metabolic pathway, phenolic A-ring estrogens are metabolized in rat liver microsomes partially to the corresponding quinols by cytochrome P450 isoenzymes. We found that these quinols could, in turn, undergo reduction to regenerate the parent estrogens consumed during the metabolic process. Among the tested endogenous reducing agents, NADH and especially NADPH produced a significant extent of reductive conversion. Enzymes available in rat liver microsomes further catalyzed this reaction with 6.5 ± 1.5 nmol · min^{-1} · (mg of protein)$^{-1}$ measured as the initial rate of estrone formation at 37°C, whereas the initial rate of second-order reaction for the reduction of E1-quinol by a 10-fold excess of NADPH in a microsome-free buffer solution and under identical incubation conditions was 0.62 ± 0.03 nmol · min^{-1}. The quinol route is, therefore, unique among estrogen-metabolizing pathways for its bioreversibility due to the facile regeneration of the phenolic A-ring estrogens consumed in the preceding oxidative process.

Estrogen quinols have been known for decades among organic chemists (Gold and Schwenk, 1958), although their metabolic formation has only been reported very recently (Ohe et al., 2000). Estrone (E1[1])- and estradiol (E2)-derived quinols [10β-hydroxy-1,4-estradiene-3,7-dione (E1-quinol) and 10β,17β-dihydroxy-1,4-estradiene-3-one (E2-quinol)] were detected from the respective estrogens during metabolic oxidation catalyzed by several cytochrome P450 isoenzymes in rat liver microsomal systems. Contrary to the well known catechol metabolites of estrogens (Zhu and Conney, 1998), quinols possess no aromatic A-ring; thus, their (bio)chemistry should be substantially different from that of catechols. Although Ohe et al. (2000) suggested quinol formation as a potential contributor to the carcinogenecity of estrogens, actual studies have shown no significant toxicity by estrogen-derived quinols (Solaja et al., 1996; Milic et al., 2001).

Incidentally, we recently found that the cytochrome P450 mimic, 5,10,15,20-tetrakis(pentafluorophenyl)-21*H*,23*H*-porphine Fe(III)/3-chloroperbenzoic acid system (Higuchi and Hirobe, 1996), yielded E1-quinol as a principal product from E1, instead of the expected catechols (L. Prokai, K. Prokai-Tatrai, and P. Perjesi, unpublished results). Quinols are obtained chemically from the corresponding *para*-substituted phenols mostly via metal-catalyzed oxidative dearomatization (Yamada et al., 1974); however, it is possible to convert the quinols back to the parent compounds through reduction by, e.g., Zn/acetic acid (Gold and Schwenk, 1958). Therefore, we hypothesized that such a reaction for estrogen-derived quinols may also be possible with endogenous reducing agent(s), and enzymes in the liver may further catalyze this process. This proposed pathway would, then, be unique, because it would allow for the regeneration of phenolic A-ring estrogens from the metabolites. A facile regeneration would also explain why quinols might be unable to accumulate as major products upon metabolism by liver microsomes, yet the corresponding route may represent a significant part in the overall estrogen-metabolizing process in the system. In this study, we tested the hypothesis for the existence of reductive conversion in enzyme-free and rat liver microsomal incubations using a representative substrate (E1-quinol), as shown in Fig. 1.

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[1] Abbreviations used are: E1, estrone; E2, estradiol; E1-quinol, 10β-hydroxy-1,4-estradiene-3,7-dione; E2-quinol, 10β,17β-dihydroxy-1,4-estradiene-3-one; LC, liquid chromotography; APCI, atmospheric-pressure chemical ionization; MS, mass spectrometry; GSH, glutathione; G-6-P, glucose-6-phosphate.

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Experimental Procedures

Materials. E1 was purchased from Steraloids Inc. (Newport, RI). E1-quinol was prepared from E1 by the method of Solaja et al. (1996). Cytochrome P450 reductase (EC 1.6.2.4) and all other chemicals were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Male rat liver microsomes (Sprague-Dawley strain, untreated) were purchased from XenoTech LLC (Kansas City, KS).

Incubation of E1-Quinol with Endogenous-Reducing Agents. E1-quinol (0.1 mM) and 1.0 mM NADH, NADPH, sodium ascorbate, or GSH in 0.1 M sodium phosphate buffer (1 ml of final volume, pH 7.5) was incubated at 37°C. At incremental time points, 100-μl aliquots were removed into ice-cooled centrifuge tubes, and 50 μl of glacial acetic acid was added. After immediate extraction with ethyl acetate (2 × 500 μl), the organic layer was evaporated under nitrogen stream at room temperature. Reconstitution of the samples with the LC mobile phase was followed by LC/MS analyses. For the control experiment, no reducing agent was used.

Microsomal Incubations of E1-Quinol. The incubation mixture (final volume 1 ml) containing 0.4 mM of $NADP^+$, 60 mM of KCl, 4 mM of $MgCl_2$, 4 mM of G-6-P, 5 U of G-6-P dehydrogenase, and 0.1 mM E1-quinol in sodium phosphate buffer (pH 7.5) was preincubated for 2 min at 37°C before microsomes (1 mg/ml protein) and 0.4 U of cytochrome P450 reductase (Roy and Liehr, 1988) were added. After 2.5 min of incubation, aliquots (100 μl) were taken, and glacial acetic acid (50 μl) was added to stop the reaction. To the mixture, ethyl acetate (3 × 500 μl, vortexed for 1 min, centrifuged at 10,000 rpm for 5 min) was added. The

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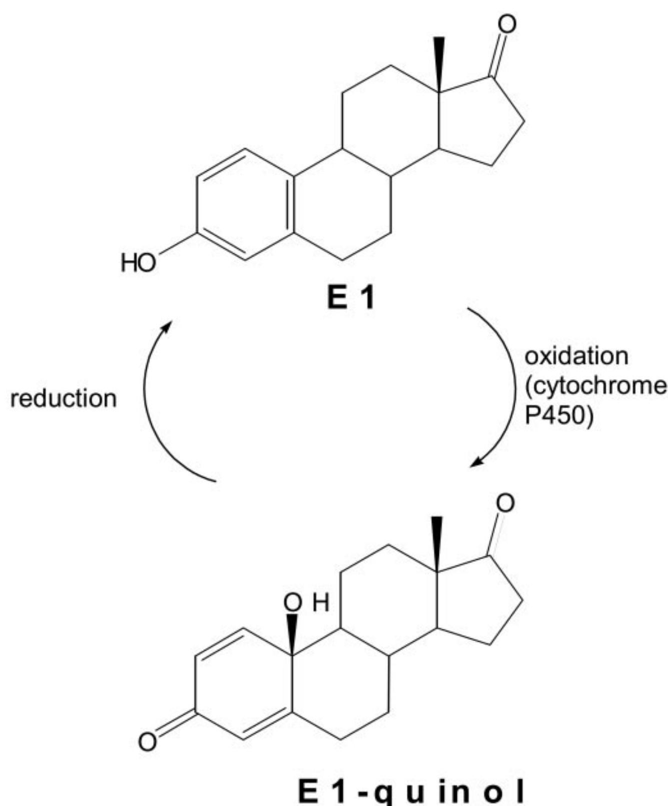

FIG. 1. *Redox conversion of E1-quinol and E1.*

organic layers were removed and combined followed by evaporation under nitrogen stream. The residue was dissolved in the mobile phase for LC/MS analysis.

Qualitative Analysis. LC separation was done using a 5 cm × 2.1 mm i.d. Discovery HS C_{18} reversed-phase column (Supelco, Bellefonte, PA) with 0.25 ml/min water/methanol/2-propanol/acetic acid/dichloromethane (53:35:5:5:2, v/v) as a mobile phase. The sample residues were dissolved in 40 μl of mobile phase, respectively, and 5 μl of the solution was injected for analysis. Mass spectra were recorded on a quadrupole ion-trap instrument (LCQ; ThermoFinnigan, San Jose, CA) using positive-ion APCI as the method of ionization. MS/MS and MS^3 product-ion scans were obtained after collision-induced dissociation with helium as the target gas. Comparison with authentic reference compound (retention time, t_R, and mass spectra) was used for unambiguous identification of E1.

Quantitative Analysis. As an internal standard, 1,3,5(10)-estratrien-17α-ethynyl-17β-ol (0.3 μM) was added before each sample extraction. E1 and E1-quinol levels were determined by LC/APCI-MS/MS and calibration with solutions of known concentrations of E1 (0.02 to 11 μM) and E1-quinol (0.2 to 125 μM) extracted for analyses. The chromatographic peak areas for E1, E1-quinol, and the internal standard were obtained from the *m/z* 271 → 253, *m/z* 287 → 269, and *m/z* 279 → 133 + 159 MS/MS transitions, respectively. Kinetic analysis of the microsomal metabolism was done by nonlinear curve-fitting (Scientist for Windows; MicroMath, Inc., Salt Lake City, UT) presuming consecutive first-order processes in the cascade of E1-quinol → E1 → E1-metabolites.

Results and Discussion

Among the many methods for steroidal quinol synthesis, we selected the "one-pot" phenol-to-quinol transformation to prepare the substrate with 3-chloroperbenzoic acid as an oxidant and dibenzoyl peroxide as the radical initiator under visible light irradiation that produced 10 β-quinol in excellent yield in refluxing aprotic solvent (Solaja et al., 1996). The quinol was meticulously purified by column chromatography (silica gel, dichloromethane, and dichloromethane/ethyl acetate, 8.5:1.5) to ensure that no contamination from E1 was present. Although Ohe et al. (2000) used gas chromatography/MS system for the detection of quinol in rat liver microsomes, we developed an LC/MS assay, because gas chromatography/MS gave us unreliable results due to artifacts detected during the analysis of the underivatized and also of the trimethylsilylated standard. Among the possible ionization techniques compatible with routine LC/MS, APCI is the method of choice for the ionization of nonpolar analytes such as steroids (Ma and Kim, 1997). The selection of E1 as a parent compound for quinol preparation was beneficial for the LC/MS, because positive-ion APCI-MS properties of E1 were more advantageous than those of E2, which showed extensive water loss (−18 u) from the protonated molecule (MH^+). With the LC/MS method developed, we have also shown the formation of E1-quinol upon incubation of estrone with rat liver microsomes under the conditions employed by Ohe et al. (2000).

To probe the hypothesis for the in vitro reductive regeneration of estrogens from quinols, we incubated E1-quinol in phosphate buffer (pH 7.5) supplemented with various endogenous reducing agents, such as ascorbic acid, GSH, NADH, or NADPH and in a rat liver microsomal system. When E1-quinol (100 μM) was treated with a 10-fold molar excess GSH, we failed to detect E1—similarly to the control experiment where no potential reducing agent was used. Only trace amount of E1 was obtained even after 12 h of incubation upon the addition of ascorbic acid. However, formation of E1 was clearly detectable even after a short period of time, when the incubation was carried out in the presence of NADH and, especially, NADPH (Fig. 2). The presence of E1 in the incubation sample was unequivocally determined by matching retention time and mass spectra (MS/MS and MS^3 product-ion spectra) to authentic E1, as shown in Fig. 2. Although the exact mechanism of the reaction is yet to be elucidated, our results clearly show that enzymes are not ultimately necessary for the reductive aromatization of estrogen-derived quinol metabolites.

When rat liver microsomal incubation of E1-quinol was done, the quinol-to-phenol transformation progressed very rapidly. The initial rate of estrone formation at 37°C was 6.5 ± 1.5 nmol · min^{-1} · (mg of protein)$^{-1}$, whereas the initial rate of the second-order reaction for the reduction of E1-quinol to estrone by a 10-fold excess of NADPH in a microsome-free buffer solution and under identical incubation conditions was 0.62 ± 0.03 nmol · min^{-1}. Consequently, while reduction of E1-quinol to E1 takes place in the mere presence of NAD(P)H, enzymes available in the liver apparently catalyze this reaction. The involvement of flavoprotein reductases distinct from the cytochrome P450 hemoprotein has been suggested in the metabolism of structurally similar steroids in mammalian tissue (Vaz et al., 1995); however, further studies are needed to address the nature of the catalyst present in the microsomal system.

In summary, our results imply a novel metabolic cycle for phenolic A-ring estrogens, which proceeds through quinols that are then regenerated to the parent estrogen consumed during preceding metabolic process by enzyme-catalyzed NAD(P)H-dependent reduction. Additionally, while redox cycling between catechol estrogen and their quinones produces reactive oxygen species (Roy and Liehr, 1988; Liehr and Roy, 1990; Nutter et al., 1994; Bolton et al., 2000), it is highly unlikely that steroidal quinols show similar properties to catechol estrogens in this regard. The cyclic dienone structure would preclude the formation of "semiquinol" radicals crucial to the prooxidant effect. Further studies are needed, however, to associate biological functions with the newly identified

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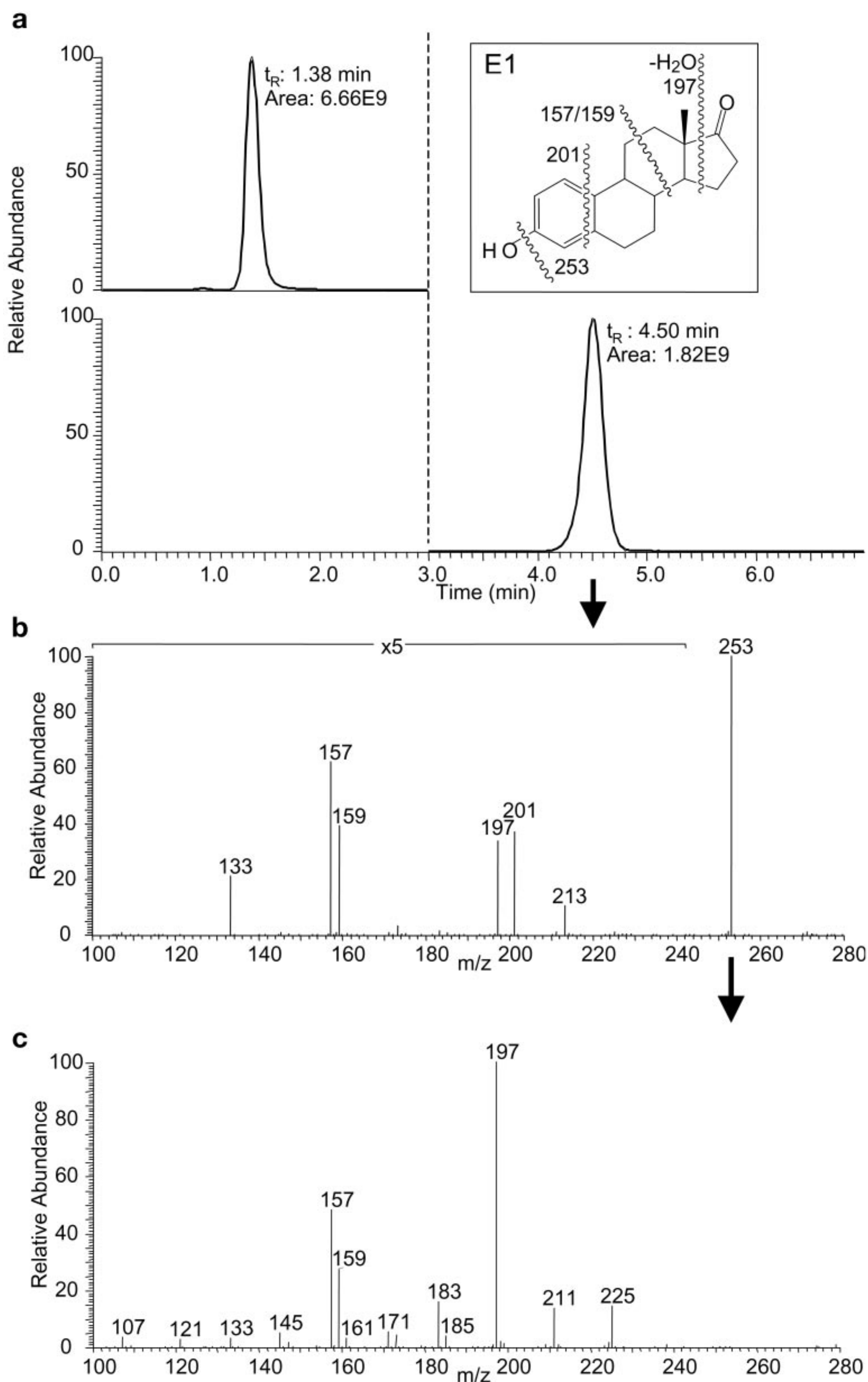

FIG. 2. *LC/APCI-MS/MS analyses showing the reduction of E1-quinol to E1 by NADPH.*

a, LC/MS/MS traces for E1-quinol (*m/z* 287 as precursor, top trace) and E1 (*m/z* 271 as precursor, bottom trace) from a sample obtained after the incubation of E1-quinol (100 μM) with 10-fold excess of NADPH in phosphate buffer (pH 7.5), with peak areas given in ion counts; b, MS/MS (*m/z* 271 as precursor); and c, MS^3 recording (*m/z* 253 selected as precursor after MS/MS) from the chromatographic peak at t_R = 4.5 min (t_R, both b and c spectra were identical to those of an authentic E1 standard). Inset, proposed origin of major fragment ions in the mass spectra b and c.

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and apparently very efficient metabolic regeneration of estrogens via quinols.

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References

Bolton JL, Trush MA, Penning TM, Dryhurst G, and Monks TJ (2000) Role of quinons in toxicology. *Chem Res Toxicol* **13:**135–160.

Gold AM and Schwenk E (1958) Synthesis and reaction of steroidal quinols. *J Am Chem Soc* **80:**5683–5687.

Higuchi T and Hirobe M (1996) Four recent studies in cytochrome P450 modelings: a stable iron porphyrin coordinated by a thiolate ligand; a robust ruthenium porphyrin-pyridine *N*-oxide derivatives system; polypeptide-bound iron porphyrin; application to drug metabolism studies. *J Mol Catal A* **113:**403–422.

Liehr JG and Roy D (1990) Free radical generation by redox cycling of estrogens. *Free Radic Biol Med* **8:**414–423.

Ma YC and Kim HY (1997) Determination of steroids by liquid chromatography–mass spectrometry. *J Am Soc Mass Spectrom* **8:**1010–1020.

Milic DR, Kop T, Juranic Z, Gasic MJ, and Solaja BA (2001) Synthesis and antiproliferative activity of epoxy and bromo compounds derived from estrone. *Bioorg Med Chem Lett* **11:**2197–2200.

Nutter LM, Wu YY, Ngo EO, Sierra EE, Gutierrez PL, and AbulHajj YJ (1994) An *o*-quinone form of estrogen produces free radicals in human breast cancer cells: correlation with DNA damage. *Chem Res Toxicol* **7:**23–28.

Ohe T, Hirobe M, and Mashino T (2000) Novel metabolic pathway of estrone and 17β-estradiol catalyzed by cytochrome P-450. *Drug Metab Dispos* **28:**110–112.

Roy D and Liehr JG (1988) Temporary decrease in renal quinone reductase activity induced by chronic administration of estradiol to male syrian hamsters. Increased superoxide formation by redox cycling of estrogen. *J Biol Chem* **263:**3646–3651.

Solaja BA, Milic DR, and Gasic MJ (1996) A novel m-CPBA oxidation: *p*-quinols and epoxides from phenols. *Tetrahedron Lett* **37:**3765–3768.

Vaz ADN, Chakraborty S, and Massey V (1995) Old yellow enzyme: aromatization of cyclic enones and the mechanism of a novel dismutase reaction. *Biochemistry* **34:**4246–4256.

Yamada Y, Hosaka K, Sanjoh H, and Suzuki M (1974) Oxidation of para-alkylphenols with thallium triperchlorate in aqueous media - effective synthesis of para-quinols. *J Chem Soc Chem Commun* **16:**661–662.

Zhu BT and Conney AH (1998) Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* **19:**1–27.

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