Identification of Fatty Acid Hydroperoxide Cofactors in the
Cytochrome P450-mediated Oxidation of Estrogens to
Quinone Metabolites

ROLE AND BALANCE OF LIPID PEROXIDES DURING ESTROGEN-INDUCED CARCINOGENESIS*

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Lipid hydroperoxide-supported metabolic redox cycling between diethylstilbestrol (DES) or catechol-estrogens and their corresponding quinones has been postulated previously to play a role in estrogen-induced toxicity and carcinogenesis. As part of an examination of this postulate, we investigated (a) the effectiveness of various lipid hydroperoxides as oxidants in microsomal-mediated quinone formation, and (b) the dependence of DES oxidation to quinone on lipid hydroperoxide levels in liver and kidney of hamsters. Kinetic analyses of the lipid hydroperoxide-mediated conversion of DES to DES quinone revealed that of the peroxides tested (9Z,11E,13(S))-13-hydroperoxoeycosatriaetraen-1-oic acid was the most powerful oxidant \( V_{max} = 10.5 \text{ nmol min}^{-1}; K_m = 21.3 \text{ \mu M} \) followed by \( (6S,6E,8Z,11Z,14Z)-5\text{-hydroperoxyeicosatetraen}-1\text{-oic acid} \) \( V_{max} = 1.0 \text{ nmol min}^{-1}; K_m = 10.0 \text{ \mu M} \). The other pure fatty acid hydroperoxide isomers or mixtures had a lower affinity for the enzyme \( K_m \) values ranging from 23.1 to 130 \text{ \mu M} \) and/or lower maximal velocity \( V_{max} \) values ranging from 0.2 to 7.0 nmol min\(^{-1}\). Levels of Z,Z-dieniestrol, which arises from spontaneous rearrangement of DES quinone, were not affected in liver of hamsters treated with estradiol implants for 9 days and an injection of 20 mg/kg DES but increased in kidney by 120\% over levels observed in animals without implants (5.5 and 12.1 nmol/g of wet tissue, respectively). Z,Z-Dienestrol concentrations correlated directly with lipid hydroperoxide concentrations in liver and kidney of control and estradiol-treated hamsters. The increased conversion of DES to quinone in kidney compared with that in liver was catalyzed by comparable activities of cytochrome P450 1A, the enzyme family catalyzing this oxidation. These results demonstrated that the oxidation of DES to quinone was directly dependent on the lipid hydroperoxide levels in the organ of metabolic conversion in addition to the enzyme activity catalyzing this reaction. The elevated lipid hydroperoxide levels in kidney of estrogen-treated hamsters indicate enhanced estrogen quinone formation and therefore enhanced generation of free radicals by metabolic redox cycling of estrogens in this organ prior to the appearance of malignancy.

Organic hydroperoxide-dependent oxidations of xenobiotics catalyzed by cytochrome P450 enzymes often result in products structurally different from those formed with NADPH as cofactor (1, 2). For instance, benz[a]pyrene is oxidized predominantly to quinone metabolites with cumene hydroperoxide (CuOOH) as cofactor, whereas mainly epoxides, dihydriodols, and phenols are formed in the presence of NADPH (1–3). This difference in product profile has been postulated to arise from the involvement of different enzymes (2) and from different reaction mechanisms in organic hydroperoxide- versus NADPH-supported reactions (4, 5).

The majority of hydroperoxide-dependent oxidations catalyzed by cytochrome P450 enzymes have been carried out with CuOOH as cofactor (1–4, 6–8). In addition, other organic and inorganic oxidants have been tested in incubations with microsomes, such as t-butylhydroperoxide, LAOOH, iodosobenzene, NaIO\(_3\), and NaClO\(_3\) (4, 6, 9). All of these oxidizing agents, except LAOOH, are abiotic synthetic chemicals, which cannot be physiological cofactors of cytochrome P450-mediated oxidations. Moreover, in LAOOH-dependent reactions, an LHP mixture has been used without further identification of the isomer(s) responsible for supporting this enzyme-mediated reaction (6). Thus, the structures of LHP oxidants, which might serve as physiological cofactors of cytochrome P450-catalyzed reactions, are not known and have been investigated in this study. In our study, mixtures of hydroperoxides of linoleic, arachidonic, and linolenic acids and also of various pure hydroperoxide isomers have been investigated with respect to their oxidizing ability and have been compared with that of CuOOH, the oxidant used most often in microsomal reactions. As substrate and test system for the evaluation of the oxidizing potential of LHP, the oxidation of DES to DES Q was chosen for several reasons. (a) This oxidation by cytochrome P450, specifically by cytochrome P450 1A1, has been studied previously, and reaction conditions are well described (10, 11). This enzyme is inducible by \( \beta \)-naphthoflavone and inhibitable by \( \alpha \)-naphthoflavone (12). (b) The product, DES Q, almost quantitatively rearranges in a spontaneous reaction to the more stable DIES, which serves as a marker for the appearance of malignancy.

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product of this oxidation (13). (c) The conversion of DES to DES Q represents the oxidation step of a metabolic redox cycling system (structures of DES, DES Q, and the redox cycling system are shown in Fig. 1) (10, 11). This redox cycling between these two stilbenes or between catechol estrogens and their corresponding quinones has been postulated to play a role in estrogen-induced kidney carcinogenesis in Syrian hamsters (10, 11), because it is a process of generation of potentially mutagenic free radicals (14). Therefore, the LHP-dependent oxidation of estrogens to quinones may represent a metabolic activation of estrogens resulting in the generation of potentially mutagenic free radicals, which possibly play a role in tumor induction. As part of our examination of this postulate, we tested in microsomal incubations the oxidizing ability of specific LHP, which might serve as physiological cofactors in cytochrome P450-dependent oxidations of estrogens to quinones. Moreover, we examined LHP levels and resulting estrogen quinone formation in kidneys of Syrian hamsters treated with estradiol and in untreated controls to assess the extent of metabolic redox cycling of estrogens preceding renal tumorigenesis. We also assayed renal activities of cytochrome P450 1A1, the enzyme family catalyzing the oxidation of estrogen to quinones (11) and the effects of α-naphthoflavone, a specific inhibitor of this enzyme (12), on enzyme activity and on LHP levels and DES formation. Values were compared with those in liver which does not develop tumors under these conditions. In our studies, we demonstrated increases in LHP levels and quinone formation in the kidney but not in liver of hamsters in response to estrogen treatment.

MATERIALS AND METHODS

Chemicals—DES, 2- and 4-hydroxyestradiol, CuOHN, linoleic acid, arachidonic acid, linolenic acid, 4,4'-isopropylidenediphenol, soybean lipoygenase type V, rescurfyn, and 7-ethoxyresorufin were purchased from Sigma. DIES, DES Q, 2,3-estradiol quinone, and 3,4-estradiol quinone were prepared as described previously (13). a-Naphthoflavone was obtained from Alrich. (9Z,11E,13S)-13-hydroxyoctadecadien-1-ol acid, 9(S)-HpODE, 13(S)-HpODE, 5(S)-HpETE, 12(S)-HpETE, and 15(S)-HpETE were purchased from Cayman Chemical Co., Ann Arbor, MI. N0-bis (trimethylsily1) trifluoroacetamide containing 1% trimethylchlorosilane was purchased from Pierce Chemical Co. LHP levels were assayed using a peroxide determination kit, purchased from Kiami Biomedical Co., Thousand Oaks, CA.

Instrumentation—UV spectra were recorded with a Hewlett-Packard spectrophotometer, model 8450 A. High pressure liquid chromatography (HPLC) analyses were carried out using a Waters Associates (Milford, MA) instrument equipped with an automated gradient controller, a model 490 multchwavelength detector, and a Waters Resolve 5-μm spherical C18 column (3.9 X 150 mm) at room temperature. Stilbenes were separated by a linear gradient from 38 to 82% aqueous methanol for 25 min at a flow rate of 1 ml/min. Under these conditions, retention times were 15.2 min for 4,4'-isopropylidenediphenol (the internal standard), 17.2 min for DES, 17.9 min for DIES, and 18.4 min for 3-DES. DIES concentrations were calculated from peak height ratios of DIES and the internal standard.

Lipid Hydroperoxides in Estrogen Metabolism

Preparation of Microsomes—Liver and kidney microsomes of male Syrian hamsters and male Sprague-Dawley rats (6 weeks old) were prepared by differential centrifugation as described previously (15). The microsomal specific contents of cytochromes P450 and b6 were determined by the method of Omura and Sato (16).

Preparation of LHP—LAAOOH, AA0OH, and LLAOOH were prepared as described by Teng and Smith (17). Briefly, 25 mg of linoleic acid was dispersed in 0.5 ml of ethanol in a 20-ml test tube. Phosphate buffer (0.1 M, 2.5 ml, pH 7.4) saturated with oxygen and containing 36,700 units of lipoygenase was added, mixed well, and incubated at 22 °C for 1 h. The preparation was acidified to pH 2.0 and extracted (three times) with 15 ml of diethyl ether. The combined diethyl ether extracts were concentrated in a flow of nitrogen. The hydroperoxides were isolated by thin layer chromatography. Yields of LAOOH were approximately 10% as determined by using 14C-labeled linoleic acid as a tracer. LAAOOH, AA0OH, and LLAOOH concentrations were determined by recording absorbance units at a wavelength of 234 nm.

Microsomal Oxidations—Oxidations of DES or catechol estrogens were carried out as described previously (10, 11). Briefly, reaction mixtures consisted of 10 mM phosphate buffer, pH 7.2, 0.2-0.2 mg/ml rat liver microsomal protein, 0-100 μM substrate (DES, 2-, or 4-hydroxyestradiol), and 0-900 nM organic hydroperoxide in a final volume of 1.0 ml. Incubations were carried out for 10-120 min at room temperature. The reactions were stopped by adding cold diethyl ether and 1 mg of sodium chloride. 4,4'-Isopropylidenediphenol (20 μg) was added to each test tube as an internal standard.

UV Monitoring of Oxidations of Estrogens to Their Quinones—Quinone formation from DES, 2-, or 4-hydroxyestradiol mediated by rat liver microsomes was monitored by the increase in UV absorption in the range of 260-460 nm recorded at timed intervals as described previously (10, 11). The identity of the quinones was confirmed by matching their UV spectra with the absorption characteristics of DES and DES Q. The oxidation of DES to DES Q was catalyzed mainly by cytochrome P450 IA1 (1), whereas the reduction of the quinone is catalyzed mainly by NADPH-dependent cytochrome P450 reductase (2) (10, 11). The semiquinone intermediate was shown in brackets because its formation is postulated but has not yet been demonstrated. It may react with molecular oxygen and form superoxide radicals (14). DES Q almost quantitatively rearranges to DIES in a spontaneous reaction and thus serves as marker of oxidation (10, 13). The reduction of DES Q forms preferentially the Z-form of DES. The equilibrium between E- and Z-forms of DES proceeds sufficiently slowly so that Z-DES can serve as a marker of reduction.

Fig. 1. Redox cycling between DES and DES Q. The oxidation of DES to DES Q is catalyzed mainly by cytochrome P450 IA1 (1), whereas the reduction of the quinone is catalyzed mainly by NADPH-dependent cytochrome P450 reductase (2) (10, 11). The semiquinone intermediate is shown in brackets because its formation is postulated but has not yet been demonstrated. It may react with molecular oxygen and form superoxide radicals (14). DES Q almost quantitatively rearranges to DIES in a spontaneous reaction and thus serves as marker of oxidation (10, 13). The reduction of DES Q forms preferentially the Z-form of DES. The equilibrium between E- and Z-forms of DES proceeds sufficiently slowly so that Z-DES can serve as a marker of reduction.
authentic estrogen quinone standards.

Analysis of DIES—DIES, the marker product of DES Q, formed by a spontaneous and almost quantitative rearrangement of the quinone (13), was extracted from each in vitro incubation mixture with diethyl ether (three times) and analyzed by HPLC as described above.

The DIES concentrations of hamster liver or kidney were determined by a thin layer chromatography/HPLC-linked procedure. Weighed tissue samples (0.2 g) were homogenized in 0.1 M phosphate buffer, pH 9.3 (1:4, w/v). Homogenates were extracted three times with 15 ml of diethyl ether. The combined diethyl ether extracts were brought to dryness in a flow of nitrogen. The concentrated was dissolved in 200 μl of chloroform and chromatographed on C18 reversed phase thin layer plates using 100% methanol. Stilbene estrogen bands were identified with the help of authentic standards, transferred to a clean 20-ml test tube, and extracted again with 15 ml of diethyl ether (twice). Extracts were concentrated in a stream of nitrogen, redissolved in 300 μl of methanol, and analyzed by HPLC as described above.

Analysis of (9Z,11E,13(S))-13-Hydroxyoctadecadien-1-0ic acid—The reaction mixture for the oxidation of DES consisted of 10 mM phosphate buffer, pH 7.2, 0.2 mg/ml rat liver microsomal protein, 40 nM 13(S)-HpODE as a cofactor, and varying concentrations of DES substrate. After incubating for 10 min at room temperature, the reaction mixtures were extracted twice with 5 ml of diethyl ether. The combined extracts were concentrated in a stream of nitrogen. The residues were dissolved in 5 μl of pyridine, 20 μl of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane was added, and the mixture was incubated for 30 min at room temperature. A 4-μl aliquot of each mixture was analyzed by gas chromatography. The gas chromatographic analysis of the reaction products was carried out using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and interfaced with a 3393A integrator. A bonded-phase DB-17 fused-silica capillary column (30 × 0.25 mm) from J&W Scientific (Rancho Cordova, CA) was used. The gas chromatographic conditions were helium carrier gas (0.72 ml/min flow rate), nitrogen as makeup gas (30 ml/min flow rate), split injection at a ratio of 50:1, detector temperature 280 °C, initial column temperature 100 °C, temperature gradient of 30 °C/min from 100 to 180 °C, 4 °C/min from 180 to 260 °C, isothermal period at 260 °C for 10 min; injector temperature 280 °C. 7-Methyl-estradiol was used as an internal standard. Under these conditions, retention times were 15.6 min for (9Z,11E,13(S))-13-hydroxyoctadecadien-1-0ic acid, 22.5 min for 7-methyl-estradiol. Hydroxycacid concentrations were calculated from peak height ratios of the product and the internal standard.

Determination of LHP Levels—Plasma, collected in test tubes containing heparin, liver, or kidney (0.2 g) were homogenized in isopropanol (1:3). Samples were centrifuged at 3,000 rpm for 15 min. LHP was assayed in the supernatant by the following method (18, 19). LHP was reduced by 10-μM N,N-methylecarbamoyl-3,7-dimethylamino-10H-phenothiazine to form LHPQ, which has about 100-fold greater absorption at 500 nm. Reaction mixtures, contained 0.05 M Tris-HCl buffer, pH 7.5, 25 mM magnesium chloride, 17 μM 7-ethoxyresorufin, and 0.8 μM rat liver microsomal protein, in a final volume of 2 ml. The reaction was initiated by the addition of 1.25 mM NADPH and allowed to proceed for 5 min at 37 °C. Reactions were stopped by addition of 1.5 ml of methanol and rapid cooling in ice. The tubes were centrifuged at 1,500 × g at 4 °C for 15 min. The UV absorbance of supernatants was measured at 572 nm and compared with those of resorufin standards at various concentrations. The cytochrome P450 1A1 activity was expressed as nmol of resorufin/mg of protein/min.

Statistical Analysis—The differences in LHP and DIES levels in tissues of estrogen-treated hamsters versus controls were analyzed by Student's t test.

RESULTS

Oxidation of Estrogens to Quinones by Microsomes and LHP—The rates of oxidation of DES, 2-, or 4-hydroxyestradiol to DES Q or catecholestrogen quinones, respectively, by rat liver microsomes and differing organic hydroperoxide co-factors were monitored by matching UV spectra of products with those of reference standards. With 0.9 mM CuOOH as co-factor, estrogen quinones were formed from DES and 2- and 4-hydroxyestradiol as described previously (10, 11) (data not shown). Comparable oxidation rates were obtained with much lower concentrations of LAOOH as co-factor (Fig. 2). Oxidation rates were highest for DES, lowest for 2-hydroxyestradiol, with an intermediate value for 4-hydroxyestradiol, when comparable incubation conditions were used. The LAOOH-dependent microsome-mediated formation of DIES, the spontaneous rearrangement product of DES Q (13) and marker of oxidation of DES to the quinone, was dependent on reaction time (Fig. 3A) and on concentrations of microsomal protein (Fig. 3B).

Large variations in rates of DES oxidation were detected depending on the nature of the LHP cofactor (Table I). For instance, comparable rates of oxidation of DES to DES Q were obtained with either LAOOH or AAOOH mixtures as oxidants over a range of 2 to 300 nM (Table I). Oxidation rates were maximal with approximately 300 nM LAOOH or AAOOH mixtures, whereas 80 nM LLAAOH sufficed for optimal rates. When LHP concentrations were higher than those required for optimal oxidation of DES to quinone (>800 nM LAOOH or AAOOH or >200 nM LLAAOH), rates of oxidation decreased and reached nondetectable levels at approximately 900 nM LHP presumably because of inactivation of the enzyme by the LHP as shown previously (11). With pure 9(S)-HpODE as cofactor, oxidation rates were lower than with identical concentrations of LAOOH mixture,

![Fig. 2. Oxidation of DES (panel A), 2-hydroxyestradiol (panel B), or 4-hydroxyestradiol (panel C) to respective quinone metabolites by liver microsomes and LAOOH. Estrogens (100 μM) were incubated with 167 μg of rat liver microsomal protein and 40 nM LAOOH. The oxidation of hydroquinones to quinones was monitored by UV spectroscopy as described previously (10, 11). The lowest absorbances were recorded at time 0. Increases in absorbance were recorded every 2 min for DES (panel A), every 20 min for 2-hydroxyestradiol (panel B), and every 10 min for 4-hydroxyestradiol (panel C). There was no increase in absorbance in the absence of cofactor and enzyme (data not shown). Rates of increase in absorbance were calculated to be 5.8 × 10⁻⁴, 0.4 × 10⁻⁴, and 1.0 × 10⁻⁴ units/min for the formation of DES Q, 2,3-estriadiol quinone, and 3,4-estriadiol quinone, respectively.](image-url)
whereas 13(S)-HpODE was much more effective as an oxidant than the LAOOH mixture (Table I). Using 2 nM 13(S)-HpODE, 3.0 nmol/ml stilbene substrate was converted to DIES, and plateau oxidation rates were achieved with an 80 nM oxidant concentration. The lowest concentration of 13(S)-HpODE required for detectable DES oxidation was 0.2 nM, resulting in 0.5 nmol/ml DIES (data not shown). At low concentrations, there was a linear correlation between consumption of 13(S)-HpODE and the formation of DIES. The formation of (9Z,11E,13(S))-13-hydroxyoctadecadien-1-oic acid, the reduction product of the hydroperoxy acid, in these incubations was determined by a gas chromatographic assay. The initial rates of consumption of 13(S)-HpODE and formation of the hydroxyacid were 26.9 and 19.1 pmol/mg of protein/min, respectively.

In contrast, CuOOH concentrations approximately 2 orders of magnitude higher than fatty acid hydroperoxides were required for comparable oxidation rates. There was no detectable product formation with 200 nM CuOOH. With 300 and 600 nM CuOOH, 1.2 and 2.0 nmol/ml DES, respectively, were converted to DIES.

In summary, mixtures of hydroperoxides of linoleic, linolenic, or arachidonic acids or pure 9(S)- or 13(S)-HpODE are more effective by approximately 2 orders of magnitude than CuOOH as oxidants supporting the microsome mediated-oxidation of DES to its quinone metabolite.

**Kinetic Analysis of the Effectiveness of LHP Oxidants**—For a full characterization of the effectiveness of various LHP mixtures or pure isomers as oxidants in the conversion of estrogens to quinones, the formation of DIES was measured as a function of DES concentration. The results were analyzed by double-reciprocal plot to obtain kinetic data for these oxidations (Figs. 4 and 5). Rates of DIES formation as a function of substrate concentration were highest with 13(S)- HpODE, lowest with 9(S)-HpODE, and intermediate with LAOOH mixture as oxidants (Fig. 4). The kinetic analysis by double-reciprocal plot revealed that 13(S)-HpODE was the most effective oxidant with very high affinity for the enzyme (defined as 1/Km), the highest vmax and the highest ratio of Vmax/Km of any of the LHP examined (Table II).

In the presence of low substrate concentrations, microsome-mediated DIES formation was higher with 5(S)-HpETE than that with the AAOOH mixture (Fig. 5). However, the ratios of these rates were reversed in the presence of ≥30 μM DES concentrations possibly because of differences in stability of these hydroperoxides. Oxidation rates were intermediate with 12(S)-HpETE and low with 15(S)-HpETE. According to the kinetic analyses, 6(S)-HpETE was an excellent oxidant in the conversion of DES to DIES, resulting in the highest affinity of DES for the enzyme (Km = 10.0 μM) (Table II).

In summary, large variations were detected in the effectiveness of various hydroperoxide isomers as oxidants in the metabolic conversion of DES to its corresponding quinone. Of the hydroperoxides tested, optimal reaction rates were obtained with 13(S)-HpODE, the most potent cofactor for this oxidation, followed by those with 5(S)-HpETE as oxidant.

**Dependence of DES Oxidation on LHP Levels in Vivo**—As part of our examination of the biological significance of LHP as oxidants in metabolic quinone formation, LHP levels were assayed and correlated with DIES formation, the marker

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**TABLE I**

| LHP       | LAOOH nmol/ml | AAOOH nmol/ml | LLAOOH* nmol/ml | 9(S)-HpODE nmol/ml | 13(S)-HpODE nmol/ml | CuOOH nmol/ml |
|-----------|--------------|---------------|-----------------|--------------------|---------------------|---------------|
| 0.0       | < 0.1        | < 0.1         | < 0.1           | < 0.1              | < 0.1               | < 0.1         |
| 2.0       | 0.6 ± 0.4    | 0.4 ± 0.1     | < 0.1           | 0.5 ± 0.1          | 3.0 ± 0.4           | < 0.1         |
| 20.0      | 1.8 ± 0.5    | 2.0 ± 0.2     | 1.6 ± 0.4       | 0.7 ± 0.1          | 4.9 ± 0.5           | < 0.1         |
| 40.0      | 2.2 ± 0.8    | 2.6 ± 0.4     | 7.2 ± 1.3       | 2.4 ± 0.5          | 6.0 ± 0.5           | < 0.1         |
| 200.0     | 3.1 ± 0.8    | 4.2 ± 0.3     | 4.1 ± 0.8       | 3.0 ± 0.6          | 7.0 ± 0.9           | < 0.1         |
| 300.0     | 4.7 ± 0.8    | 6.0 ± 1.3     | 2.7 ± 0.3       | 3.6 ± 0.6          | 7.3 ± 0.2           | 1.2 ± 0.1     |
| 600.0     | 3.4 ± 0.4    | 4.0 ± 0.5     | < 0.1           | 4.4 ± 0.6          | 6.0 ± 0.2           | 2.0 ± 0.1     |
| 900.0     | < 0.1        | < 0.1         | < 0.1           | < 0.1              | < 0.1               | 4.5 ± 0.9     |

*Approximately 4.1 ± 0.8 nmol/ml DIES was formed when 40 nM LLAOOH was used as cofactor.
product of DES Q, in kidney and liver of hamsters treated with estradiol and/or α-naphthoflavone and in untreated controls (Fig. 6). LHP levels were determined in liver, kidney (Fig. 6), and plasma (data not shown) and compared with blank values to help eliminate the possibility of lipid perox-

![Image](https://example.com/image.png)

**FIG. 4.** Effectiveness of mixtures of LAAOOH (open circles), LLAAOOH (filled circles), and of pure 9(S)-HpODE (open triangles) and 15(S)-HpODE (filled triangles) as oxidants in the microsome-mediated oxidation of DES to DES Q. Incubation mixtures contained indicated concentrations of DES, 0.2 mg of rat liver microsomal protein, and 40 nM LHP. DES Q was allowed to rearrange spontaneously to DIES, which was extracted and assayed by HPLC. Product formation was plotted as a function of substrate concentration (inset on left) or as a double-reciprocal plot. Values are the means of three experiments.

![Image](https://example.com/image.png)

**FIG. 5.** Effectiveness of a mixture of AAOOH (open circles) or pure 15(S)-HpETE (filled triangles), 12(S)-HpETE (filled circles), and of 5(S)-HpETE (open triangles) as oxidants supporting the microsome-mediated oxidation of DES to DES Q. Incubation mixtures contained various concentrations of DES, 0.2 mg of rat liver microsomal protein, and 40 nM LHP. DES Q was allowed to rearrange spontaneously to DIES, which was extracted and assayed by HPLC. Product formation was plotted as a function of substrate concentration (inset on left) or as a double-reciprocal plot. Values are the means of three experiments.

**TABLE II**

Kinetic parameters of the oxidation of DES to DES Q catalyzed by rat liver microsomes and various LHP mixtures or pure hydroperoxide isomers

| Cofactors | V_max | K_m | V_max/K_m |
|-----------|-------|-----|-----------|
| LAAOOH    | 7.0   | 100.0 | 0.07      |
| 9(S)-HpODE| 6.5   | 23.1 | 0.02      |
| 15(S)-HpODE| 10.5  | 21.3 | 0.49      |
| AAOOH     | 6.1   | 130.0 | 0.05      |
| 5(S)-HpETE| 1.0   | 10.0 | 0.10      |
| 12(S)-HpETE| 2.4   | 110.0 | 0.02     |
| 18(S)-HpETE| 0.2   | 25.4 | 0.01      |
| LLAOOH    | 2.1   | 51.0 | 0.04      |

**FIG. 6.** Panel A, LHP concentrations in liver (left) and kidney (right) of male Syrian hamsters treated with estradiol implants for 9 days (filled bars), in untreated controls (open bars), in hamsters treated with estradiol implants for 9 days and α-naphthoflavone for the last 3 days of the experiment (left hatched bars), and in hamsters treated with α-naphthoflavone for 3 days (right hatched bars). Hamsters were treated with estradiol implants as described previously (22, 27). LHP concentrations were determined by UV absorbance of methylene blue, which was formed by LHP-mediated oxidation of 10-N-methylcarbamoyl-5,7-dimethyloxamino-10H-phenothiazine. Values are the means of 10 samples ± S.D. in two experiments (**p < 0.001). Panel B, DIES concentrations in liver (left) and kidney (right) of male Syrian hamsters treated with estradiol implants for 9 days (filled bars), in untreated controls (open bars), in hamsters treated with estradiol implants for 9 days and α-naphthoflavone for the last 3 days of the experiment (left hatched bars), and in hamsters treated with α-naphthoflavone for 3 days (right hatched bars). Hamsters were treated with estradiol implants as described previously (22, 27). On the 9th day, each hamster received 20 mg/kg DES dissolved in propylene glycol by intraperitoneal injection. After 30 min, the animals were killed, and DIES concentrations were measured in livers and kidneys by a thin layer chromatography/HPLC-linked procedure. Values are the means ± S.D. of two experiments each with five hamsters/group (n = 10, **p < 0.01). Panel C, DIES concentrations in liver in relation to LHP concentrations in these organs. LHP concentrations were taken from panel A and DIES concentrations from panel B and are presented on the vertical and horizontal axes, respectively: O, control liver; ♂, liver of estradiol-treated hamsters; Δ, control kidney; ▲, kidney of estradiol-treated hamsters; ☑, liver of α-naphthoflavone-treated hamsters; ◊, kidney of α-naphthoflavone-treated hamsters; ♦, liver of estradiol plus α-naphthoflavone-treated hamsters;  ▪, kidney of estradiol plus α-naphthoflavone-treated hamsters.

oxidation by autoxidation during sample preparation. In untreated hamsters, renal LHP levels exceeded those in liver by 61% (Fig. 6A). The chronic treatment of hamsters with estradiol for 9 days significantly increased LHP levels by 44 and 217% over controls in kidney and in plasma, respectively,
whereas those in liver remained unchanged. These results are consistent with previous reports of a significant increase in fluorescent products of lipid peroxidation in kidney but not in liver of estradiol-treated hamsters (22). In hamsters treated with either α-naphthoflavone or α-naphthoflavone plus estradiol, LHP levels in liver were decreased by approximately 69 and 72% and in kidney by 57 and 95%, respectively, from control levels. When a single injection of DES was administered to control hamsters, DIES metabolite concentrations in liver were approximately half of those in kidney (Fig. 6B). In animals exposed to chronic estradiol treatment followed by injection of an identical dose of DES, DIES concentrations in liver remained unchanged from control values, whereas those in kidney were significantly increased by 120%. In hamsters treated with either α-naphthoflavone or α-naphthoflavone plus estradiol, DIES concentrations were decreased in liver by 96% and in kidney by 81–84% from control levels. DIES metabolite concentrations in liver and kidney of estradiol and/or α-naphthoflavone-treated hamsters and in controls were also plotted as a function of LHP concentrations in these organs (Fig. 6C). There was a linear relationship between DIES and LHP concentrations. These results demonstrate that LHP levels are directly dependent on the availability of cytochrome P450 IA family enzymes (to catalyze redox cycling) and of estrogens as substrates for the redox cycling. They also demonstrate that the oxidation of DES to quinone in vivo is directly related to the local LHP level in the organ of metabolic conversion and on cytochrome P450 IA family enzymes which catalyze the oxidation. Thus, the oxidation of estrogens to quinone metabolites in vivo depends on the availability of hydroperoxides as physiological cofactors for the cytochrome P450-mediated oxidation.

The increased metabolic conversion of DES to quinone in kidney of controls and estradiol-treated hamsters compared with liver described above was not caused by increased enzyme activity (Fig. 7). Activities of both renal and hepatic cytochrome P450 IA, the enzyme family catalyzing the oxidation of estrogens to quinone metabolites (11), in estradiol-treated hamsters were approximately 0.43 and 0.49 nmol/mg of protein/min, respectively, which were unchanged from values in controls (Fig. 7B). These values of cytochrome P450 IA activity were comparable in liver and kidney of control or estrogen-treated hamsters, although specific contents of total cytochrome P450 in liver were much higher than corresponding values in kidney (Fig. 7A). Both cytochrome P450 IA activities and specific contents of cytochrome P450 in liver and kidney of α-naphthoflavone- or α-naphthoflavone plus estradiol-treated hamsters were significantly decreased as was expected for this inhibitor of cytochrome P450 IA family enzymes. The specific contents of cytochrome b6, which has previously been shown to enhance the cytochrome P450-mediated oxidation of estrogens to quinones (23), were significantly decreased in liver by 70–84% in response to estradiol and/or α-naphthoflavone treatment. Changes of values in kidney were statistically not significant in response to estradiol but were decreased by α-naphthoflavone alone or in combination with the hormone (Fig. 7C).

In summary, LHP levels in liver or kidney of control or estradiol-treated hamsters correlated directly with the extent of metabolic oxidation of DES to its corresponding quinone. This correlation was obtained with comparable values of activities of cytochrome P450 IA, the enzyme family catalyzing this oxidation, in liver and kidney of control and estradiol-treated hamsters. Taken together, these data demonstrate that LHP served as physiological cofactor for the cytochrome P450 IA-mediated quinone formation from DES in vivo as demonstrated above with microsomal enzymes and various pure unsaturated fatty acid hydroperoxides.

**DISCUSSION**

Unsaturated fatty acid hydroperoxides are highly effective oxidants in the cytochrome P450-mediated conversion of hydroquinones to quinones. The CuOOH-dependent microsomal formation of quinone from DES, 2-, or 4-hydroxyestradiol (10, 11) or from benzo[a]pyrene (1–3) has been described previously. However, unsaturated fatty acid hydroperoxides are much more effective oxidants than CuOOH by approximately 2 orders of magnitude. The very low levels of LHP necessary for the oxidation of estrogens to quinones suggest that these reactive substances likely are physiological cofactors of this metabolic reaction. This postulate is supported by the fact that (a) LHP levels in liver or kidney of control or estradiol-treated hamsters are approximately in the range required for effective oxidation of hydroquinones to quinones, and (b) an excellent correlation exists between levels of LHP and DIES, the spontaneous rearrangement product of DES Q (13), in liver and kidney of control and estradiol-treated hamsters that each received a single dose of DES. The exact structure of the physiological LHP cofactor(s) required for supporting cytochrome P450 IA-dependent oxidations has not
yet been identified in vivo and requires further study. In vitro, large variations exist between various fatty acid hydroperoxides and isomers with respect to their effectivenss as oxidants in cytochrome P450-dependent oxidations. This differential effectivenss of LHP isomers as metabolic oxidants may be mainly because of their chemical and metabolic stabilities.

The mechanism of the LHP-dependent cytochrome P450 IA-mediated oxidation of estrogens to corresponding quinones is unknown. Homolytic cleavage of hydroperoxides by cytochrome P450 enzymes has been postulated previously to result in alkoy radicals which may be the oxidizing species (24, 25). This mechanism is consistent with the LHP-mediated oxidation of estrogens. An alkoy radical may react with DES or catecholestrogens to form an estrogen semiquinone and a hydroxylated fatty acid. The semiquinone may then be converted to a quinone in a second analogous oxidation.

The LHP-dependent cytochrome P450 IA-mediated oxidation of DES or catecholestrogens to corresponding quinones represents the oxidative step of a metabolic redox cycle (10, 11) (illustrated in Fig. 1 and Scheme 1). The estrogen quinones are reduced in this cycle to DES or 2- or 4-hydroxyestradiol by NADPH-dependent cytochrome P450 reductase or NADH-dependent cytochrome b, reductase (10, 23). The semiquinone intermediates may react with molecular oxygen to form oxygen radicals, which may damage cellular macromolecules (14). Various types of oxygen free radical damage, such as an increased 8-hydroxydeoxyguanine content of DNA (26), an increased carbonyl content of proteins (27), and increases in renal LHP levels as shown in this report and in fluorescent products of lipid peroxidation (23), have been observed in hamsters treated chronically with estrogen. Thus, LHP plays a dual role in kidneys of estrogen-treated hamsters: it is consumed in this redox cycle as oxidant in the cytochrome P450 IA-dependent oxidation of estrogens to quinones, and at the same time, LHP may be formed by the interaction of unsaturated fatty acids with reactive radicals, generated by metabolic redox cycling as reviewed by Kappus (28). The balance of LHP consumption and generation may depend on (a) the availability of estrogens or other endogenous or exogenous hydroquinone substrates for redox cycling; (b) the activities of the cytochrome P450 IA family of enzymes catalyzing the oxidation to quinone; and (c) the activities of detoxifying enzymes and their abilities to detoxify reactive oxygen species and active radicals.

In normal, healthy cells, cytotoxic LHP may be deactivated by redox cycling and may be consumed as oxidants for the conversion of hydroquinones to quinones, which in turn may be detoxified by other more efficient pathways. For instance, adequate activities of free radical-detoxifying enzymes have been demonstrated in hamster liver and may prevent an increase in LHP in this organ even when estrogen, a substrate for redox cycling, is supplied to the animals (29). In contrast, control hamster kidney, an organ prone to develop estrogen-induced cancer, contains LHP levels higher than liver, presumably because of constitutively high cytochrome P450 IA activity (4, 570 and 470 pmol/nmol P450/min in kidney and liver, respectively). In addition, renal activities of detoxifying enzymes (glutathione peroxidase, glutathione transferase, quinone reductase, catalase, and superoxide dismutase) are lower than those in liver by up to 1 order of magnitude (14, 29). The increase in renal LHP levels by the enhanced supply of estrogen substrate for metabolic redox cycling may in turn enhance quinone formation, free radical generation by redox cycling, and free radical-mediated covalent damage to DNA and proteins specifically in the kidney, the target of hormonal cancer. This cell toxicity in combination with hormone-dependent cell transformation and growth may be required for full development of estrogen-induced tumors.

In summary, 13(S)-HpODE and 5(S)-HpETE have been identified as effective oxidants and likely physiological cofactors supporting the oxidation of hydroquinones to quinones by cytochrome P450 enzymes, illustrated in this report with estrogen substrates. The administration of estradiol to hamsters resulted in enhanced formation of LHP and thus enhanced in vivo oxidation of DES to DES Q specifically in the kidney, the target of estrogen-induced carcinogenesis. Enhanced LHP formation by redox cycling of estrogens and subsequent free radical generation is postulated to play a role in estrogen-induced kidney tumorigenesis.

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REFERENCES

1. Capdeville, J., Estabrook, R. W., and Prough, R. A. (1980) Arch. Biochem. Biophys. 206, 186-195
2. Wone, A. K., Saddie, E., and Ragan, E. (1986) Biochem. Pharmacol. 35, 1553-1568
3. Cavaliere, E., Wong, A., and Ragan, E. (1987) Biochem. Pharmacol. 36, 435-440
4. Vaz, A. D. N., and Coon, M. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1172-1176
5. Guenzel, F. P. (1991) J. Biol. Chem. 266, 10019-10022
6. Hrycay, E. G., Gustafsson, J.-Å., Ingelman-Sundberg, M., and Ernster, L. (1976) Eur. J. Biochem. 61, 43-52
7. Berge, C. H., and Coon, M. J. (1981) J. Biol. Chem. 256, 12127-12133
8. Cavaliere, E. L., Ragan, E. O., Cremonesi, P., and Devanesan, P. D. (1988) Biochem. Pharmacol. 37, 2173-2182
9. Cavaliere, J.-Å., and Bergman, J. (1978) FEBS Lett. 70, 276-280
10. Liehr, J. G., Ulden, A. A., and Strobel, H. W. (1986) J. Biol. Chem. 261, 16685-16690
11. Roy, D., Bernhardt, A., Strobel, H. W., and Liehr, J. G. (1992) Arch. Biochem. Biophys. 296, 450-456
Lipid Hydroperoxides in Estrogen Metabolism

12. Blank, J. A., Tucker, A. N., Sweatlock, J., Gasiewicz, T. A., and Luster, M. I. (1987) Mol. Pharmacol. 32, 168-172
13. Liehr, J. G., DeGue, B. B., Ballatore, A. M., and Henkin, J. (1983) Biochem. Pharmacol. 32, 3711-3715
14. Roy, D., and Liehr, J. G. (1988) J. Biol. Chem. 263, 3646-3651
15. Dignam, J. D., and Strobel, H. W. (1977) Biochemistry 16, 1116-1123
16. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378
17. Teng, J. I., and Smith, L. L. (1985) J. Chromatogr. 350, 445-451
18. Tateishi, T., Yoshimine, N., and Kuzuya, F. (1987) Exp. Gerontol. 22, 103-111
19. Ohishi, N., Ohkawa, H., Miike, A., Tatano, T., and Yagi, K. (1985) Biochem. Pharm. 34, 1039-1051
20. Pohl, R. J., and Fouts, J. R. (1980) Anal. Biochem. 107, 150-155
21. Klotz, A. V., Stiegman, J. J., and Walsh, C. (1984) Anal. Biochem. 140, 136-145
22. Roy, D., and Liehr, J. G. (1992) Mol. Cell. Biochem. 110, 31-39
23. Roy, D., Strobel, H. W., and Liehr, J. G. (1991) Arch. Biochem. Biophys. 285, 331-338
24. Coon, M. J., and Vaz, A. D. N. (1987) J. Biol. Chem. 262, 35-40
25. Vaz, A. D. N., Roberts, E. S., and Coon, M. J. (1989) in Oxygen Radicals in Biology and Medicine (Simon, M. G., Taylor, K. A., Ward, J. F., and von Sonntag, C., eds) pp. 501-507, Plenum Publishing Corp., New York
26. Roy, D., Floyd, R. A., and Liehr, J. G. (1991) Cancer Res. 51, 3882-3885
27. Winter, M. L., and Liehr, J. G. (1991) J. Biol. Chem. 266, 14446-14450
28. Kappas, H. (1986) in Oxidative Stress (Sies, H., ed) pp. 273-310, Academic Press, London
29. Roy, D., and Liehr, J. G. (1989) Cancer Res. 49, 1475-1480