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Microsomal Hydroxylation of 2- and 4-Fluoroestradiol to Catechol Metabolites and Their Conversion to Methyl Ethers: Catechol Estrogens as Possible Mediators of Hormonal Carcinogenesis

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SUMMARY

In male Syrian hamsters, an animal model for estradiol-induced carcinogenesis, 2-fluoroestradiol was not carcinogenic, whereas 4-fluoroestradiol induced kidney tumors after a prolonged latency period, compared with estradiol (100% tumor incidence), when the compounds were administered to hamsters in hormonally equipotent doses. Catechol estrogen metabolites have previously been postulated to mediate this estrogen-induced kidney carcinogenesis. To examine this proposed mechanism of tumor induction by estrogens, we investigated the conversion of 2- and 4-fluoroestradiol to catechol metabolites by kidney and liver microsomes of hamsters and the further conversion to methyl ethers by catechol-*O*-methyltransferase, and we compared the values with those obtained with nonfluorinated estrogens as substrates. The rates of conversion of 2-fluoroestradiol to 2-hydroxyestradiol and 2-fluoro-4-hydroxyestradiol by hepatic microsomes were 30–50% lower than corresponding rates with estradiol as substrate. With renal microsomes the rate of 4-hydroxylation was 10 times faster than that of estradiol, whereas 2-hydroxylation was at best marginal. With 4-fluoroestradiol as substrate the rate of 2-hydroxylation by hepatic microsomes was enhanced 5-fold, compared with values for estradiol, but 4-hydroxyestradiol formation was almost eliminated. In contrast, the conversion of this substrate to 4-fluoro-2-hydroxyestradiol by kidney microsomes occurred at a rate 15 times faster than 2-hydroxylation of estradiol, whereas 4-hydroxyestradiol formation proceeded at a rate of 315 pmol/mg of protein/min. Except for the decrease in both 2- and 4-hydroxylation of 2-fluoroestradiol by liver microsomes, fluorine substitution of estrogenic phenols enhanced microsome-mediated aromatic hydroxylation at sites unoccupied by substituents. At pH 7.5, the highest rates of catechol-*O*-methyltransferase-mediated methylation were observed with the catechol metabolites of 2-fluoroestradiol, 2-fluoro-4-hydroxyestradiol and 2-hydroxyestradiol (3780 and 2960 pmol/mg of protein/min, respectively). Lower rates were found with those of 4-fluoroestradiol, 4-fluoro-2-hydroxyestradiol and 4-hydroxyestradiol (1670 and 470 pmol/mg of protein/min, respectively). These data are consistent with the postulate that catechol metabolites of estrogens are reactive intermediates in estrogen-induced carcinogenesis. For the noncarcinogenic 2-fluoroestradiol, a high metabolic flux was observed through a pathway of renal 2-fluoro-4-hydroxyestradiol formation and further conversion to methyl ethers. This flux likely results in low steady state concentrations of catechol metabolites in kidneys of hamsters treated with this modified estrogen and therefore in its lack of carcinogenic activity. In contrast, the carcinogenic activity of 4-fluoroestradiol is consistent with its rapid conversion in the kidney to 2- and 4-hydroxylated metabolites and a less rapid methylation of these catechols.

Estrogenic hormones, such as E2 or estrone, induce kidney tumors in 80–100% of hamsters chronically treated with these steroids (1). In contrast, ethinylestradiol is only weakly carcinogenic in this animal model for hormonal cancer, despite the potent estrogenic activity of this synthetic steroid (1, 2). This lack of correlation between hormonal potency of estrogens and their capacity for tumor induction has led to the postulate that, in addition to receptor-mediated events, metabolic processes are required for the induction of tumors in this species by estrogenic hormones (3, 4). Specifically, the conversion of estrogens to CE in the target organ for carcinogenesis has been postulated as a necessary step for tumor induction, because these metabolites are capable of undergoing metabolic redox cycling (5), a process generating potentially mutagenic free radicals (6). As part of our examination of this hypothesis, both 2FE2 and 4FE2 were synthesized in the hope that fluorine substituents on estrogens would inhibit metabolic hydroxyl-

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ABBREVIATIONS: E2, estradiol; 2FE2, 2-fluoroestradiol; 4FE2, 4-fluoroestradiol; 2OHE2, 2-hydroxyestradiol; 4OHE2, 4-hydroxyestradiol; 2F4OHE2, 2-fluoro-4-hydroxyestradiol; 4F2OHE2, 4-fluoro-2-hydroxyestradiol; COMT, catechol-*O*-methyltransferase; CE, catechol estrogen(s); RAL, relative adduct labeling, as defined by Reddy and Randerath (22); TMS, trimethylsilyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ation of the aromatic A-ring at the site of substitution because of the high carbon-fluorine bond strength (7). In line with this hypothesis, 2FE2 did not induce kidney tumors in hamsters, whereas 4FE2 was as carcinogenic as E2 but required a longer latency period than the natural hormone when these estrogens were administered to hamsters in hormonally equipotent doses (8, 9). However, the rates of conversion of 2FE2 or 4FE2 to catechol metabolites by hamster liver or kidney microsomes were reported to equal or exceed those of E2 when measured by radioenzymatic assay (10–12). These measurements were conducted without the use of the appropriate CE standards, 2F4OHE2 or 4F2OHE2, because it was reported that they could not be synthesized from the fluorinated parent estrogens by the commonly used Fremy's salt procedure of oxidation of phenols (13). Moreover, the COMT-coupled radioenzymatic assay, used previously for measuring rates of catechol formation from E2 and fluorinated estrogens, had not been validated and was found by us to underestimate CE formation by 2–3 orders of magnitude, compared with a validated product isolation assay (14, 15). Therefore, the reported apparent lack of correlation between the tumor incidence rates of 2FE2 and 4FE2 and their rates of conversion to catechol metabolites prompted us to re-examine these metabolic rates for the fluorinated estrogens by validated product isolation assay (16) and to compare them with values obtained with E2 as substrate. The rates of catechol formation were assayed with microsomes of hamster kidney, a target of estrogen-induced tumorigenesis, and compared with those of liver, where tumors are not induced under these conditions. Authentic reference standards, 2F4OHE2 and 4F2OHE2, were synthesized for accurate measurements. In addition, we assayed the rates of conversion of CE to noncarcinogenic methoxyestrogens by COMT, because reactive catechol metabolites are inactivated by COMT-mediated methylation. Our data indicate that catechol metabolites of 2FE2 may be formed and further catabolized at high rates, which may prevent their accumulation and carcinogenic interaction with cellular macromolecules.

Materials and Methods

Chemicals. 2FE2 was a gift of Dr. John Ward of Eli Lilly Research Laboratories (Indianapolis, IN). Porcine liver COMT, E2, 2OHE2, and 4OHE2 were purchased from Sigma Chemical Co. (St. Louis, MO); Fremy's salt (potassium nitrosodisulfonate) was from Aldrich Chemical Co. (Milwaukee, WI). 4FE2 and 2-hydroxyestradiol-17-acetate were purchased from Steraloids (Wilton, NH), and heptafluorobutyric anhydride, chlorotrimethylsilane, and anhydrous triethylamine were from Pierce Chemical Co. (Rockford, IL). All reagents and solvents were reagent grade and were used without further purification. Melting points of CE were determined on an Electrothermal melting point apparatus.

Microsomal preparation. Six-week-old male Syrian hamsters were purchased from Harlan Sprague Dawley (Houston, TX). Liver and kidney microsomes of 10 animals were prepared by the method of Dignam and Strobel (17).

Microsomal incubation conditions. The conditions for microsomal incubation, derivatization of estrogens, and gas chromatographic analysis were as described by Roy *et al.* (16). The incubation mixture consisted of 2 mg/ml microsomal protein for liver microsomes or 4 mg/ml for kidney microsomes, 5 mM NADPH, 50 mM HEPES-Tris buffer, pH 7.5, containing 5 mM ascorbate, and 10–100 μM E2, 2FE2, or 4FE2, in a final volume of 250 μl. Incubations were carried out at 37° for 10 min and were stopped by placing culture tubes on ice. Ice-cold 50 mM HEPES-Tris buffer (1 ml) containing 5 mM ascorbate, pH 7.5, and 2.5 μg of 2-hydroxyestradiol-17-acetate as internal standard were added. The steroids were extracted twice with 5 ml of ice-cold ethyl acetate saturated with water. The ethyl acetate layer was dried (with Na_2SO_4) and evaporated under nitrogen. Control incubations were carried out as described above but without substrate.

Derivatization. The dry extracts were dissolved in a mixture of 100 μl of ethyl acetate and 1 μl of triethylamine and were acylated by the addition of 25 μl of heptafluorobutyric anhydride. Samples were heated at 55° for 20 min, evaporated under nitrogen, and redissolved in 200 μl of ethyl acetate. A 1-μl aliquot of this solution was injected into the gas chromatograph. It is important to note that heptafluorobutyryl esters of estrogens are stable for several hours at ambient temperature but cannot be stored for longer periods of time without appreciable decomposition. The detection limit of the heptafluorobutyryl estrogens used in this study was approximately 1 pmol, in agreement with that reported previously (16).

TMS derivatives of 2F4OHE2 and 4F2OHE2 were prepared by dissolving the steroid in 1 ml of pyridine, adding 0.1 ml of chlorotrimethylsilane, and heating at 55° for 15 min. A 1-μl aliquot of this solution was injected into the gas chromatograph-mass spectrometer for analysis.

Gas chromatography. Gas chromatographic analysis of E2 and its metabolites was carried out with a Hewlett-Packard model 5890A gas chromatograph equipped with an electron-capture detector and a model 3393A integrating recorder. A bonded-phase DB-5 fused-silica capillary column (30 m × 0.25 mm i.d.) from J & W Scientific (Folsom, CA) was used. The gas chromatography conditions were 15 psi helium carrier gas head pressure, splitless injection, 280° injector temperature, and 50° initial column temperature. A temperature gradient of 30°/min from 50° to 245° was followed by a 5-min isothermal period, a second temperature rise of 1°/min to 265°, and a second 5-min isothermal period. Gas chromatographic analyses of 2FE2 and 4FE2 and their metabolites were carried out on a DB-17 column from J & W Scientific. The chromatographic conditions were the same as described above, except that a 30°/min temperature rise from 50° to 180° was followed by a 2-min isothermal period, a 10°/min rise to 245°, and a second 5-min isothermal period. Blank values were obtained from control incubations carried out without substrate estrogen. Any CE possibly contained by the microsomal preparations were below the detection limits of the assay.

Gas chromatography-mass spectrometry. Mass spectra were recorded with a Nermag model R 10–10C mass spectrometer equipped with a Hewlett-Packard model 5890A gas chromatograph. TMS derivatives of 2F4OHE2 and 4F2OHE2 were analyzed on a 30-m × 0.32-mm i.d. DB-5 column (J & W Scientific). The column temperature was programmed from 200° to 330° at 10°/min and the flow rate was 2.3 ml/min. The split ratio was 30:1 and the injection temperature was 250°.

NMR and IR spectrophotometry. NMR spectra were obtained on a JEOL GX270WB spectrophotometer. The frequency for protons was 270 MHz and for carbon was 67.9 MHz. An Attached Proton Test pulse sequence was used for the ^{13}C NMR analyses, which distinguished quaternary and methylene carbons from methine and methyl groups. IR spectra were obtained on a Mattson Instruments model 4020 Fourier transform-IR spectrophotometer.

COMT-catalyzed methylation. COMT-catalyzed methylation of CE was carried out according to the method of Roy *et al.* (18). The reaction mixture consisted of 0.01 M Tris·HCl buffer (pH 6.0–10.0), 1.2 mM magnesium chloride, 200 μM *S*-adenosylmethionine iodide (containing 10 μCi of *S*-[*methyl*-^{3}H]adenosylmethionine), 1 mM dithiothreitol, and the indicated concentrations of CE (10–100 μM), in a final volume of 0.5 ml. The reaction was started by the addition of COMT and stopped by the addition of 1.5 ml of a saturated solution of sodium borate (pH 10.0). The methoxyestrogens were extracted and counted as described previously (18).

Synthesis of 2F4OHE2 and 4F2OHE2 standards. 2F4OHE2

was prepared according to the procedure of Stubenrauch and Knuppen (19). A solution of 225 μl (3.2 mmol) of concentrated nitric acid and a few grains of sodium nitrite in 10 ml of water was added dropwise to a stirred solution of 970 mg (3.3 mmol) of 2FE2 in 30 ml of acetic acid at 40°. A 15-ml aliquot of water was added after 15 min, and the mixture was allowed to crystallize at ambient temperature overnight. Filtration of the product gave 850 mg (76%, based on 2FE2) of white crystals (m.p., 240–250°). The product, 2-fluoro-4-nitroestradiol, was used directly in the next step.

A 10-g portion of sodium hydrosulfite was added to a hot stirred solution of 850 mg (2.5 mmol) of 2-fluoro-4-nitroestradiol in 300 ml of acetone, 60 ml of water, and 60 ml of 1 N aqueous sodium hydroxide, and the mixture was heated at reflux for 15 min. The acetone was evaporated under a stream of nitrogen, and the residue was extracted with chloroform, dried (with $MgSO_4$), and evaporated. The residual oil was dissolved in 300 ml of acetic acid and added over 2 min to a rapidly stirred solution of 10 g of sodium periodate in 700 ml of 0.1 N hydrochloric acid. The resulting orange-red solution was stirred for 2 min and then extracted twice with chloroform. The chloroform extract was washed with a solution of 5 g of potassium iodide in 100 ml of water, and the mixture was then washed with 150 ml of 5% sodium bisulfite solution. The organic phase was washed with water, dried (with $MgSO_4$), and evaporated. The residual oil was chromatographed on 200 g of Woelm 63–100-μm silica gel in a glass column, and the product was crystallized twice from 10% ethyl acetate in hexane, yielding 49 mg (6%, based on 2-fluoro-4-nitroestradiol) of 2F4OHE2 [m.p., 255–259°; IR (KBr): ν_{max} 3504, 2939, 1600, 1323, and 1049 cm^{-1}; ^1H NMR (acetone-d_6): δ 0.77 (s, 3 H, C-18 CH_3), 1.0–1.8 (m, 8 H), 1.8–2.4 (m, 5 H), 2.4–2.6 (m, 1 H), 2.7–2.9 (m, 1 H), 2.98 (br s, 1 H, C-17 OH), 3.6–3.8 (m, 1 H, C-17 CH), 6.56 (d, 1 H, J = 12 Hz, C-1 CH), and 7.7–8.0 (br s, 2 H, C-3, 4 OH); ^{13}C NMR (acetone-d_6): δ 11.59 (C-18 CH_3), 23.77 and 24.03 (C-11 and -12), 27.22 and 27.62 (C-7 and -15), 31.05, 37.68, 39.41, 43.97 (C-13), 44.91, 50.98, 81.81 (C-17), 103.80 (d, J = 18 Hz, C-1), 120.14, 130.48 (d, J = 16 Hz, C-3), 133.17, 145.33, and 151.24 (d, J = 233 Hz, C-2); electron impact mass spectrometry (TMS derivatized): m/z 522 (M^+), 432 [(M − TMSOH)$^+$], 415, 391 [(M − TMSO-$CH_2CH_2CH_2$)$^+$], 337, and 129].

4F2OHE2 was prepared from 75 mg (0.26 mmol) of 4FE2, according to the procedure described above for 2F4OHE2. The yield of white crystals (m.p., 180–190°) was 6 mg (8%, based on 4FE2) [IR (KBr): ν_{max} 3399, 2930, 1767, 1458, and 1316 cm^{-1}; ^1H NMR (acetone-d_6): δ 0.78 (s, 3 H, C-18 CH_3), 1.0–1.8 (m, 5 H), 1.8–2.4 (m, 5 H), 2.6–3.2 (m, 6 H), 3.6–3.8 (m, 1 H, C-17 CH), 6.63 (s, 1 H, C-1 CH), and 6.8–7.0 (br s, 2 H, C-2, 3 OH); ^{13}C NMR (acetone-d_6): δ 11.59 (C-18 CH_3), 22.66 and 23.79 (C-11 and -12), 27.24 and 27.39 (C-7 and -15), 31.07, 37.74, 39.55, 44.01 (C-13), 44.80, 50.91, 108.17 (C-1), 115.45 (d, J = 17 Hz, C-5), 131.23 (d, J = 15 Hz, C-3), 132.09, 145.07, and 150.38 (d, J = 237 Hz, C-4); electron impact mass spectrometry (TMS derivatized): m/z 522 (M^+), 391 [(M − TMSO-$CH_2CH_2CH_2$)$^+$], 285, 269, and 147].

Each isomer was crystallized and was pure by thin layer chromatographic analysis. In addition, gas chromatographic analysis showed a purity of >99% for each product.

Oxidation of 2-FE2 by Fremy's salt procedure. The oxidation of 2-FE2 by Fremy's salt was carried out according to the method of Gelbke *et al.* (13, 20). A 160-mg (0.596 mmol) portion of Fremy's salt was added to a solution of 50 mg (0.174 mmol) of 2FE2 in 16 ml of acetone and 25 ml of 10% aqueous acetic acid, and the reaction mixture was stirred at 20° for 15 min. A second 160-mg portion of Fremy's salt was added and the reaction mixture was stirred for an additional 15 min. The solution turned violet and then yellow. Extraction with chloroform gave an organic phase, which was washed with 1 N HCl and then with water. Glacial acetic acid (3.5 ml) and potassium iodide (200 mg, 1.2 mmol) were added to the organic phase and the mixture was shaken for 3 min. Iodine was removed by extraction with 0.1 N sodium thiosulfate solution and the organic layer was washed with 1 N HCl and water and dried over sodium sulfate. The reaction product was analyzed by gas chromatography.

^{32}P-postlabeling analysis of DNA adducts. DNA was isolated from liver tissue of male Syrian hamsters as described previously (21, 22). This DNA was sheared by three passages through a 23-gauge needle and denatured by heat treatment at 100° for 15 min. Mixtures containing 1 mg of DNA, hamster liver microsomes (2 mg of microsomal protein), 1 mM cumene hydroperoxide, and 0.25 mg of CE in 2 ml of potassium phosphate buffer, pH 7.4, were incubated at 37° for 2 hr. Reaction mixtures without estrogen served as control. Each reaction was performed in triplicate. After incubation, the reaction mixtures were extracted with diethyl ether (three times) and washed with chloroform/isoamyl alcohol (24:1, v/v) (once). The DNA was precipitated with cold ethyl alcohol (three times). The DNA samples (10 μg) were digested with micrococcal nuclease and spleen phosphodiesterase and were used for DNA adduct analysis with a nuclease P1-enhanced ^{32}P-postlabeling assay as described previously (21, 22).

Results

Synthesis of 2F4OHE2 and 4F2OHE2

The fluorinated CE standards, 2F4OHE2 and 4F2OHE2, needed for the gas chromatographic assay were synthesized independently from 2FE2 and 4FE2, respectively, by a modification of the procedure of Stubenrauch and Knuppen (19). The mass spectra of the TMS derivatives contained a parent ion at m/z 522, the correct mass for the derivatized estrogens. The ^1H NMR spectrum of 2F4OHE2 contained a doublet at δ 6.56 (J = 12 Hz), indicating vicinal H-F coupling of the C-1 hydrogen. In contrast, the ^1H NMR spectrum of 4F2OHE2 showed only a singlet at δ 6.63 for the C-1 hydrogen. In addition, the ^{13}C NMR of 2F4OHE2 contained a doublet at δ 151.24 (J = 233 Hz) for C-F coupling of the C-2 carbon and doublets at δ 103.80 (J = 18 Hz) and 130.48 (J = 16 Hz) for long-range C-F coupling of C-1 and C-3. The ^{13}C NMR spectrum of 4F2OHE2 exhibited a similar coupling pattern, with doublets at δ 150.38 (J = 237 Hz), 115.45 (J = 17 Hz), and 131.23 (J = 15 Hz), corresponding to C-4, C-5, and C-3, respectively. These spectral data clearly establish the structures of the synthetic catechol standards.

An alternative synthesis of 2F4OHE2 by oxidation of 2FE2 with Fremy's salt was re-examined because it had previously been reported to result only in 2OHE2 (20). In contrast, a gas chromatographic analysis of the reaction product showed a composition of 88% unreacted 2FE2, 8% 2OHE2, and 5% 2F4OHE2, which had been missed previously. The low yield of 2F4OHE2 in this one-step procedure precluded its practical use.

Conversion of 2FE2 and 4FE2 to Catechol Metabolites

Hamster liver. CE formation by hamster liver microsomes was measured with E2 as substrate to validate the assay and to assess the effect of fluorine substituents of estrogens on aromatic hydroxylation by cytochrome P450 enzymes. The rates of conversion of E2 to 2OHE2 and 4OHE2 were 1520 and 430 pmol/mg of protein/min, respectively (Fig. 1A), which were consistent with values obtained previously by product isolation assay (14, 15). The rates of conversion of 2FE2 to 2OHE2 and 2F4OHE2 were 30–50% lower than corresponding values for CE formation from E2 as substrate (Fig. 1B). When 100 μM 4FE2 was incubated with liver microsomes, 2-hydroxylation occurred at a rate of 7500 pmol/mg of protein/min, a 5-fold faster rate, compared with E2 as substrate (Fig. 1C). In contrast, 4-hydroxylation of 4FE2 was marginal. Thus, hepatic cytochrome P450 enzymes were capable of replacing fluorine with hydroxyl in the case of 2FE2, albeit at a slower rate than

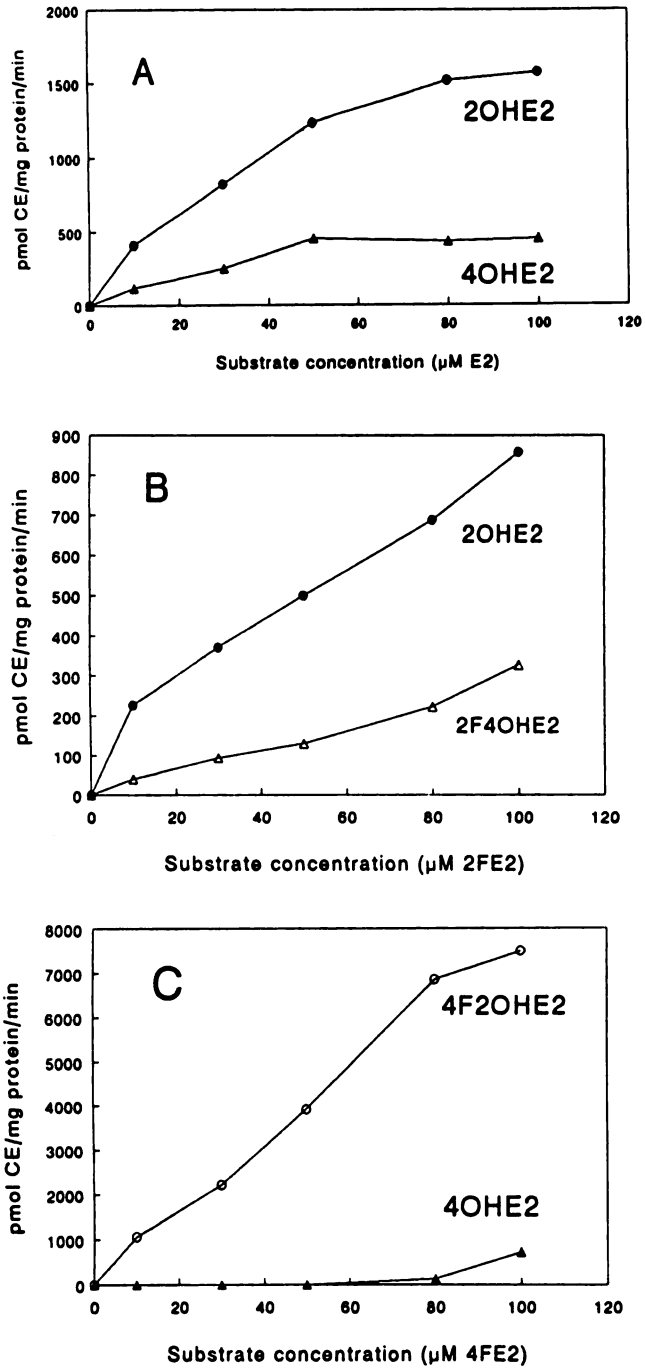

Fig. 1. Rates of NADPH-dependent 2- and 4-hydroxylations (*circles* and *triangles*, respectively) of E2 (A), 2FE2 (B), and 4FE2 (C) by microsomes isolated from livers of male Syrian hamsters. The incubation conditions are described in Materials and Methods. The reaction products were analyzed by capillary gas chromatography (16). Values are means of duplicate assays. Inter- and intra-assay variations were within 10%.

hydrogen at the C-2 position of E2, but could not overcome the strength of the C-F bond of 4FE2. In addition, the electron-withdrawing effect of fluorine on the aromatic A-ring of the steroid enhanced microsome-mediated 2-hydroxylation of 4FE2.

The kinetic values also reflected these relationships. Both the affinity (defined as $1/K_m$) of 2FE2 for the enzyme and the V_{max} for the 2- and 4-hydroxylation of 2FE2 were lower than corresponding values for E2 (Table 1). The K_m value for the 4-hydroxylation of 4FE2 could not be determined. The V_{max} value for the 2-hydroxylation of 4FE2 was 5-fold higher than that for E2, but the affinity for the enzyme was lower than observed with the parent hormone. In summary, rates of CE formation with 2FE2 as substrate were 30–50% lower than corresponding rates with E2, whereas rates of 2-hydroxylation of 4FE2 were enhanced 5-fold and 4OHE2 formation was almost eliminated.

Hamster kidney. The rates of conversion of E2 to 2OHE2 and 4OHE2 were 25 and 75 pmol/mg of protein/min (Fig. 2A), respectively, which were consistent with previous data obtained by product isolation assay (15). In contrast, 4-hydroxylation of 2FE2 occurred at a rate approximately 10 times faster (865 pmol/mg of protein/min) than that of E2, whereas 2-hydroxylation of the 2-fluorinated substrate was at best marginal (Fig. 2B). The 2-hydroxylation of 4FE2 occurred at a rate approximately 15 times faster than that of E2 (Fig. 2C). Moreover, microsome-mediated replacement of the fluorine substituent of 4FE2 by hydroxyl occurred at a rate of 315 pmol/mg of protein/min. As was observed with E2 as substrate, the rates of catechol formation from the fluorinated estrogen substrates exceeded by 1–3 orders of magnitude the values obtained by COMT-coupled radioenzymatic assay (10–12).

The kinetic data support the metabolism rates described above. The V_{max} value for the 4-hydroxylation of 2FE2 was 11 times larger than that of E2, whereas the affinity for the enzyme (defined as $1/K_m$) was lower than observed with E2 (Table 1). The V_{max} values for 2- and 4-hydroxylation of 4FE2 were 40 and 5 times larger, respectively, than values observed for E2. The corresponding K_m values were approximately double or half, respectively, those with E2 as substrate.

In summary, the conversion by hamster kidney microsomes of 2FE2 to 2F4OHE2 and of 4FE2 to 4F2OHE2 occurred at rates 10 and 15 times faster, respectively, than corresponding hydroxylations of E2. The substitution of fluorine for hydroxyl was marginal in the case of 2FE2 but proceeded at a rate of 315 pmol/mg of protein/min in the case of 4FE2. Thus, fluorine substitution of aromatic phenols enhanced microsome-mediated hydroxylation at sites unoccupied by substituents.

COMT-Catalyzed Methylation of Catechol Metabolites

The COMT-catalyzed methylation of CE metabolites was carried out to determine the relative rates of inactivation of these reactive intermediates. The pH optima were measured

TABLE 1

Kinetic parameters of the NADPH-dependent 2- and 4-hydroxylations of E2, 2FE2, and 4FE2 by microsomes isolated from livers or kidneys of male Syrian hamsters

The K_m and V_{max} values were derived from double-reciprocal plots (not shown) of the data presented in Figs. 1 and 2.

Organ, substrate	K_m (V_{max})[a]			
	2OHE2	4OHE2	4F4OHE2	4F2OHE2
		μM		
Liver				
E2	28 (1573)	26 (453)		
2FE2	39 (858)		61 (327)	
4FE2		ND[b] (719)		48 (7500)
Kidney				
E2	4.5 (25)	23 (75)		
2FE2	ND (38)		40 (865)	
4FE2		8.5 (315)		10 (1040)

[a] K_m values are given as μM and V_{max} values as pmol/mg protein/min.
[b] ND, value could not be determined from the double-reciprocal plots.

for the methylation of 2- and 4OHE2, 2F4OHE2, and 4F2OHE2 before determination of the rates of methylation. The pH optimum for COMT-catalyzed methylations was approximately pH 7.5, except in the case of 4OHE2, which was converted to methyl ethers at a maximal rate at pH 8.0–8.5 (Fig. 3A). At pH 7.5 and 30 μM substrate concentrations, the highest rates of methylation were observed with 2F4OHE2 and 2OHE2 as substrates (3780 and 2960 pmol/mg of protein/min, respectively), and lower rates were observed with 4F2OHE2 and 4OHE2 (1670 and 470 pmol/mg of protein/min, respectively). High rates of methylation of 2F4OHE2 and 2OHE2, the catechol metabolites of 2FE2, were also observed when the COMT-catalyzed methylation was recorded as a function of substrate

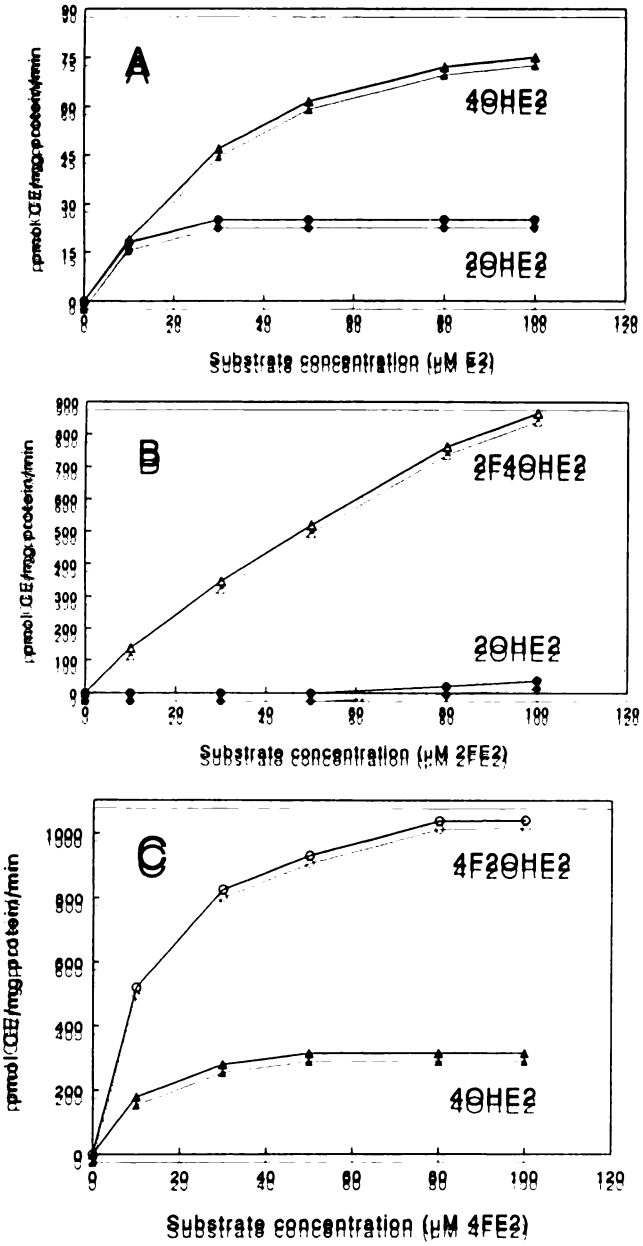

Fig. 2. Rates of NADPH-dependent 2- and 4-hydroxylations (*circles* and *triangles*, respectively) of E2 (A), 2FE2 (B), and 4FE2 (C) by microsomes isolated from kidneys of male Syrian hamsters. The incubation conditions are described in Materials and Methods. The reaction products were analyzed by capillary gas chromatography (16). Values are means of duplicate assays. Inter- and intra-assay variations were within 10%.

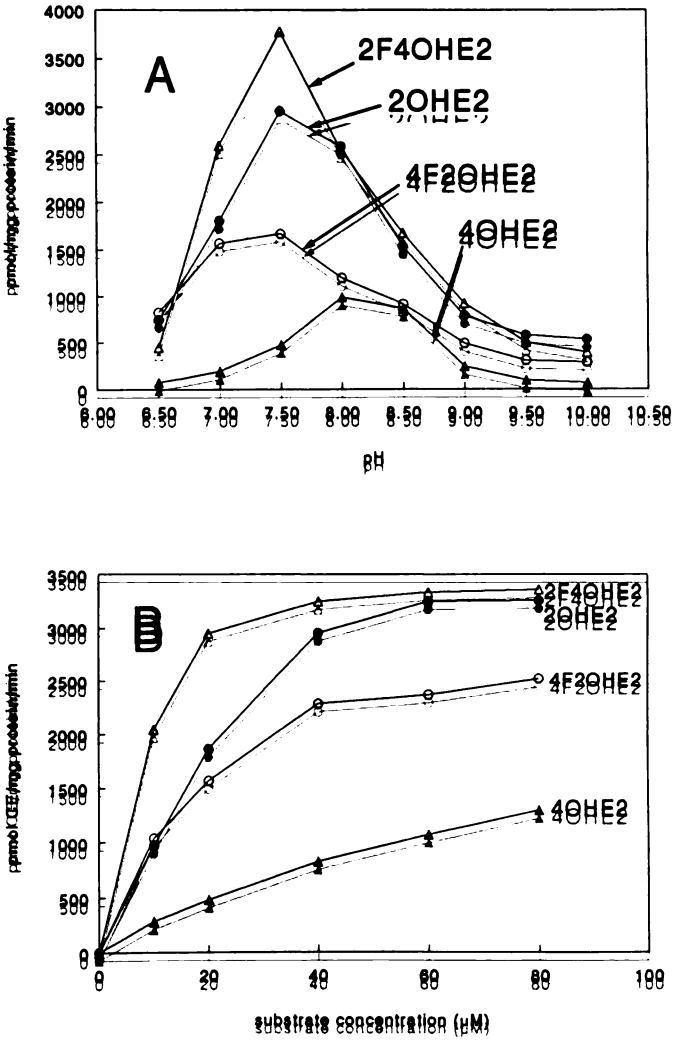

Fig. 3. Rates of COMT-mediated methylation of CE as a function of pH (30 μM substrate concentration) (A) and substrate concentration (B). The incubation conditions are described in Materials and Methods. The methylated estrogens were extracted and analyzed by liquid scintillation counting as described previously (18). Values are means of duplicate assays. Inter- and intra-assay variations were within 10%.

concentration (Fig. 3B). The rates of methylation of these two catechol metabolites (at 40 μM substrate concentration) were 3250 and 2960 pmol/mg of protein/min, respectively, whereas they were lower for the catechol metabolites of 4FE2 (2280 and 820 pmol/mg of protein/min for 40 μM 4F2OHE2 and 4OHE2, respectively). In summary, 2F4OHE2 and 2OHE2, the catechol metabolites of 2FE2, were methylated by COMT at faster rates than 4F2OHE2 and 4OHE2, the catechol metabolites of 4FE2.

DNA Adduct Formation of CE

The microsome-mediated oxidation of the fluorinated CE to quinones and the covalent DNA adduct formation of these quinones *in vitro* were examined by ^{32}P-postlabeling assay and compared with DNA adduct levels obtained with quinones of 2OHE2 and 4OHE2. After incubation with purified DNA, microsomes, and cumene hydroperoxide, 2OHE2 and 4OHE2 formed DNA adducts, which could be demonstrated by their distinct patterns on two-dimensional chromatograms (Fig. 4, b and d, respectively). Total adduct concentrations were 14.7 and 7.1×10^{-8} RAL, respectively. The fluorinated CE, after oxida-

tion to quinones, also reacted with DNA and each gave distinct adduct patterns, as shown in Fig. 4, c and e. Adduct concentrations induced by 4F2OHE2 (9.9×10^{-8} RAL) were comparable to those induced by 2OHE2. However, concentrations of total 2F4OHE2-induced DNA adducts (71.0×10^{-8} RAL) were 7-fold greater than levels obtained with 4OHE2. Because of the low levels of DNA adducts, no attempt was made to isolate and structurally identify these addition compounds.

In summary, oxidized metabolites of all four CE examined were capable of forming DNA adducts when incubated with purified DNA. Total adduct levels of 2OHE2, 4F2OHE2, and 4OHE2 ranged from 14.7 to 7.1×10^{-8} RAL, whereas those of 2F4OHE2 were 71.0×10^{-8} RAL.

Discussion

Our data confirm previous reports, obtained by radioenzymatic assay, that hamster liver or kidney microsomal enzymes are capable of replacing the halogen of modified estrogen substrates with hydroxyl (10–12). However, these previous measurements by radioenzymatic assay underestimated by at least 1 order of magnitude microsome-mediated CE formation by defluorination of the modified estrogens. Moreover, the aromatic hydroxylation of such fluorinated estrogen substrates at positions unoccupied by halogen substituents was underestimated by up to 3 orders of magnitude. The COMT-coupled radioenzymatic assay under-reported CE formation from 2FE2 and 4FE2 because (i) fluorinated CE metabolites, 2F4OHE2 and 4F2OHE2, were not used as authentic standards; (ii) the radioenzymatic assay relies on the methylation of CE products and, thus, differences in rates of methylation of CE, as shown in this investigation, may have resulted in inaccurate values of CE formation; (iii) 2OHE2 inhibits the methylation of 4OHE2 (18) and, thus, this assay under-reports the formation of 4-hydroxylated estrogens because of enzyme inhibition; and (iv) the COMT-coupled radioenzymatic assay for CE formation was not optimized, as shown previously (14).

The predominant 2-hydroxylation of E2 by hepatic microsomes has previously been postulated to be catalyzed by estradiol-2-hydroxylases, which also form small amounts of 4-hydroxylated estrogens as a result of a lack of specificity of the enzymes (23). In contrast, the predominant formation of 4OHE2 by hamster kidney microsomes at high substrate concentrations had been taken as evidence for a specific estrogen-4-hydroxylase activity in this organ, in addition to the more common hepatic-type estradiol-2-hydroxylase activity. Our data with fluorinated estrogens as substrates for hamster liver and kidney microsomal enzymes are consistent with this proposal. Hepatic estradiol-2-hydroxylase is capable of defluorinating 2FE2 to form 2OHE2 (albeit at a reduced rate, compared with E2 as substrate) but cannot overcome the C-F bond strength of 4FE2 to form 4OHE2. Conversely, kidney-specific estradiol-4-hydroxylase is also capable of replacing fluorine with hydroxyl substituents in the conversion of 4FE2 to 4OHE2 (at an elevated reaction rate, compared with E2 as substrate), whereas renal estrogen-2-hydroxylase is unable to break the C-F bond of 2FE2. The differences in rates of replacement of fluorine with hydroxyl substituents by hepatic and renal enzymes support the concept that different isoforms of cytochrome P450 catalyze 2- and 4-hydroxylation of estrogens. Fluorine substituents significantly enhance aromatic hydroxylation by either renal isoform at positions unoccupied by substituents, i.e., C-2 of 4FE2 and C-4 of 2FE2. The electron-withdrawing effect of fluorine is most likely responsible for this enhanced hydroxylation. The fluorine substituent of 4FE2 also enhances 2-hydroxylation in the liver. There is no increase in hepatic 4-hydroxylation of 2FE2, because liver does not contain a specific estrogen-4-hydroxylase and generates 4OHE2 from E2, most likely as a result of a lack of specificity of hepatic estradiol-2-hydroxylase (23).

The methylation of CE was examined because concentrations of catechol metabolites are determined by the rates of both their formation and their catabolism. Our data demonstrate that 2F4OHE2 and 2OHE2, the two catechol metabolites of 2FE2, are methylated at very rapid rates. In addition, hamster kidney microsomes convert 2FE2 to only minor amounts of 2OHE2, which might therefore not be available as an inhibitor of the COMT-mediated methylation of 4-hydroxylated estrogens (18). In contrast, 4F2OHE2 and 4OHE2, the CE formed by metabolic conversion of 4FE2, are methylated more slowly. Moreover, the methylation of 4OHE2 might be inhibited by locally generated 4F2OHE2 in the kidney. Thus, renal steady state concentrations of catechol metabolites of 2FE2 might be very low, whereas those of 4FE2 might be elevated.

Our data are consistent with the hypothesis that CE metabolites mediate estrogen-induced carcinogenesis (3, 4). 2FE2, a potent estrogen, is not carcinogenic, whereas the parent hormone E2 induces hamster kidney tumors with 100% incidence (8, 9). The lack of carcinogenic activity of 2FE2 is in agreement

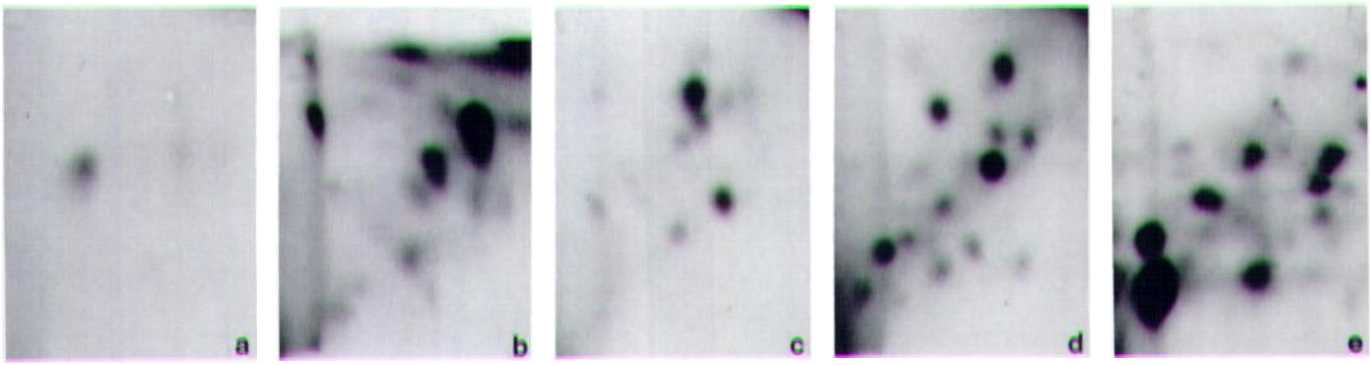

Fig. 4. ^{32}P-Postlabeling analysis of covalent DNA adducts formed by incubation of purified hamster liver DNA, hamster liver microsomes, cumene hydroperoxide, and 2OHE2 (b), 4F2OHE2 (c), 4OHE2 (d), or 2F4OHE2 (e). Incubations without catechol substrate served as controls (a). DNA digestion, labeling, chromatography, and visualization of adducts were carried out as described previously (21, 22). ^{32}P-labeled adducts were located by autoradiography for 4 hr at −80° using DuPont Lighting Plus intensifying screens and Kodak X-Omat film.

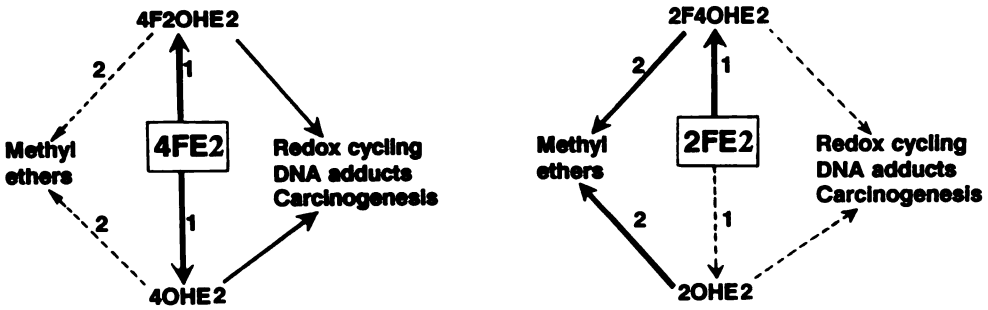

Fig. 5. Proposed pathway of catechol metabolism of 4FE2 (*left*) and 2FE2 (*right*). 4FE2 is converted to both 4F2OHE2 and 4OHE2 by hamster kidney microsomal enzymes (*1*) at elevated rates (——), compared with E2 as substrate. These catechols are methylated by COMT (*2*) at lower rates (– – –) than the catechol metabolites of 2FE2. Thus, 4OHE2 and 4F2OHE2 may be available for free radical generation by redox cycling and/or DNA adduct formation and carcinogenesis. 2FE2 is converted by hamster kidney microsomal enzymes (*1*) to 2F4OHE2 at an elevated rate (——), compared with E2 as substrate. 2OHE2 formation is marginal (– – –). Both catechols are methylated by COMT (*2*) at very rapid rates (——), compared with catechol metabolites of 4FE2. Thus, steady state concentrations of 2F4OHE2 may be insufficient for the induction of tumors by redox cycling and/or DNA adduct formation.

with its conversion by renal microsomal enzymes at a fast rate to 2F4OHE2, which is also methylated at a very fast rate. The conversion of 2FE2 to 2OHE2 as a percentage of total catechol metabolite formation is marginal in the kidney. This catechol is therefore not available as an inhibitor of the methylation of the 4-hydroxylated metabolite, as shown previously (18). Thus, there is a high metabolic flux through a pathway of catechol formation and further conversion to methyl ethers, which likely results in low steady state concentrations of catechol metabolites. In addition, the rapid metabolism of 2FE2 also decreases the hormonal stimulus exerted by the parent estrogen, which is necessary for the transformation of kidney cells and for the growth of estrogen-dependent tumors (1). Although 2F4OHE2 was the most DNA reactive of the CE tested *in vitro*, a low steady state concentration of this metabolite in hamster kidney as a result of its rapid inactivation may thus minimize DNA damage in this organ. In contrast, 4FE2, an estrogen with comparable carcinogenic activity but prolonged latency period, compared with E2, is hydroxylated at high rates at both the C-2 and C-4 positions of the aromatic ring. The resulting catechol metabolites are methylated less rapidly. In addition, the 2-hydroxylated metabolite, formed at high rates in the kidney, may inhibit the methylation of 4OHE2, as observed previously with nonfluorinated CE (18). Thus, CE metabolites of 4FE2 may persist at higher levels than those of 2FE2 or undergo DNA adduct formation or metabolic redox cycling, a mechanism for the generation of potentially mutagenic free radicals (5, 6). The prolonged latency period, compared with that observed with E2, may have been caused by the lowered hormonal stimulus of 4FE2 concentrations via enhanced metabolic conversion to catechol metabolites.

The high rate of 2-hydroxylation of 4FE2 in liver may not result in liver tumors because catechol metabolites may be detoxified much more efficiently in this organ, as evidenced by much higher detoxifying enzyme activities in liver, compared with kidney (6, 24). Moreover, high rates of 4FE2 metabolism decrease concentrations of this modified estrogen and thus its estrogenic stimulus, which is necessary for cell transformation and growth of hormone-dependent tumors.

In summary, a high metabolic flux was observed for 2FE2 through a pathway of catechol formation and further conversion to methyl ethers (illustrated in Fig. 5). This flux is likely to result in low steady state concentrations of CE metabolites in kidneys of hamsters treated with this modified estrogen and, therefore, in its lack of carcinogenic activity in this species. In contrast, the carcinogenic activity of 4FE2 is consistent with its conversion to 2- and 4-hydroxylated metabolites at rapid rates and a less rapid methylation of these CE. The rapid decrease in concentrations of 2FE2 or 4FE2, caused by rapid metabolic conversions to CE, likely results in decreased estrogenic stimuli and may contribute to the lack of carcinogenicity of 2FE2 and the prolonged latency period of 4FE2.

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