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4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: Implications for the mechanism of uterine tumorigenesis

(2-hydroxyestradiol/4-hydroxyestradiol/estrogen metabolism/cytochrome P450/uterine myoma)

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ABSTRACT **Estradiol is converted to catechol estrogens via 2- and 4-hydroxylation by cytochrome P450 enzymes. 4-Hydroxyestradiol elicits biological activities distinct from estradiol, most notably an oxidant stress response induced by free radicals generated by metabolic redox cycling reactions. In this study, we have examined 2- and 4-hydroxylation of estradiol by microsomes of human uterine myometrium and of associated myomata. In all eight cases studied, estradiol 4-hydroxylation by myoma has been substantially elevated relative to surrounding myometrial tissue (minimum, 2-fold; mean, 5-fold). Estradiol 2-hydroxylation in myomata occurs at much lower rates than 4-hydroxylation (ratio of 4-hydroxyestradiol/2-hydroxyestradiol, 7.9 ± 1.4) and does not significantly differ from rates in surrounding myometrial tissue. Rates of myometrial 2-hydroxylation of estradiol were also not significantly different from values in patients without myomata. We have used various inhibitors to establish that 4-hydroxylation is catalyzed by a completely different cytochrome P450 than 2-hydroxylation. In myoma, α-naphthoflavone and a set of ethynyl polycyclic hydrocarbon inhibitors (5 μM) each inhibited 4-hydroxylation more efficiently (up to 90%) than 2-hydroxylation (up to 40%), indicating >10-fold differences in K_i (<0.5 μM vs. >5 μM). These activities were clearly distinguished from the selective 2-hydroxylation of estradiol in placenta by aromatase reported previously (low K_m, inhibition by Fadrozole hydrochloride or ICI D1033). 4-Hydroxylation was also selectively inhibited relative to 2-hydroxylation by antibodies raised against cytochrome P450 IB1 (rat) (53 vs. 17%). These data indicate that specific 4-hydroxylation of estradiol in human uterine tissues is catalyzed by a form(s) of cytochrome P450 related to P450 IB1, which contribute(s) little to 2-hydroxylation. This enzyme(s) is therefore a marker for uterine myomata and may play a role in the etiology of the tumor.**

2-Hydroxylation of estradiol (E_2) is the primary metabolic oxidation of this hormone in most mammalian species (1, 2). In human liver, this metabolic oxidation is catalyzed mainly by cytochrome P450 IIIA and, to a lesser extent, IA family enzymes (2, 3). Cytochrome P450 IA enzymes have also been identified as estrogen 2-hydroxylases in extrahepatic tissues and in MCF-7 human breast cancer cells (2, 4, 5). In addition, aromatase has been reported to catalyze the 2-hydroxylation of E_2 in placenta (6). Aromatic hydroxylations of estrogens by these enzymes mainly result in 2-hydroxylated catechol estrogens (CE) accompanied by small amounts of 4-hydroxylated estrogens (<20% of total CE metabolites) (1, 2). Therefore, 4-hydroxyestradiol (4-OH-E_2) has been considered as an unimportant by-product of 2-hydroxyestradiol (2-OH-E_2) formation because rates of its formation by microsomal preparations are low relative to total CE formation (1, 2) and also because urinary concentrations of 4-hydroxylated estrogens are much lower than those of 2-hydroxylated metabolites (7, 8). In contrast, in microsomes of MCF-7 cells induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a specific E_2 4-hydroxylase activity distinct from the more common 2-hydroxylase activity has been detected by selective inhibition of the latter activity with anti-rat P450 IA IgG (4). Moreover, a specific E_2 4-hydroxylase activity has been identified in rat pituitary and mouse uterus, in which it represents the almost exclusive form of CE formation (9, 10), and in hamster kidney, in which it has been unmasked by inhibitors of E_2 2-hydroxylase activity, such as by Fadrozole hydrochloride (11). A physiological function of this estrogen metabolite and of the form(s) of cytochrome P450 catalyzing its formation is not known.

E_2 induces malignant cancers in hamster kidney (12), mouse uterus (13), and hyperplasia in rat pituitary (14). The expression of a specific E_2 4-hydroxylase activity in these three rodent organs, in which estrogens induce tumors, and also in a human breast cancer cell line has been taken as evidence for a role of 4-OH-E_2 formation in the development of benign and/or malignant E_2-induced tumors (11, 15). This hypothesis was developed because CE, including 4-OH-E_2, are capable of undergoing metabolic redox cycling between the catechol (hydroquinone) and corresponding quinone forms (16). Such redox cycling is a mechanism to generate potentially mutagenic free radicals (17). 4-OH-E_2 may also contribute to carcinogenesis by acting as a mitogen because it is known to be a long-acting estrogen (18). In line with this hypothesis, 4-OH-E_2 was found to be as carcinogenic as E_2 in the hamster kidney tumor model (19).

In this study, 2- and 4-hydroxylase activities have been assayed in microsomes of human uterine myoma and surrounding myometrial tissue to examine the hypothesis that 4-OH-E_2 formation serves as a marker of uterine myomata and plays a role in tumor development. Values were compared to enzyme activities in myometrial microsomes of patients without apparent myomata. For the identification of enzymes catalyzing 2- and 4-hydroxylation, several inhibitors of specific forms of cytochrome P450 were investigated. We also examined CE formation by microsomes of human placenta for the validation

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Abbreviations: E_2, 17β-estradiol; 2-OH-E_2 and 4-OH-E_2, 2-hydroxyestradiol and 4-hydroxyestradiol, respectively; CE, catechol estrogen(s).

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of the assays. In our studies, we identified 4-hydroxylation of E_2 in human tissues as a specific metabolic pathway distinct from the more common 2-hydroxylation.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: E_2, NADPH, ascorbic acid, Hepes, Tris base, and Tris·HCl from Sigma; α-naphthoflavone, a specific inhibitor of cytochrome P450 IA1/IA2 (20), from Aldrich; 2-OH-E2 and 4-OH-E_2 from Steraloids (Wilton, NH); the aromatase inhibitors Fadrozole hydrochloride (CGS 16949A) (21, 22) from A. Bhatnagar, CIBA–Geigy, and ICI D1033 (23) from M. Dukes, ICI; 1-ethynylpyrene, which preferentially inhibits cytochrome P450 IA1 (24), 1- and 2-ethynylnaphthalene, which preferentially inhibit cytochrome P450 IB1 (24), and 2-, 3-, and 9-ethynylphenanthrene, which inhibit both cytochromes P450 IB1 and P450 IA1 (24, 25), from W. L. Alworth, Tulane University (New Orleans); [6,7-^{3}H]estradiol (specific activity, 40–60 Ci/mmol; 1 Ci = 37 GBq) from Amersham; neutral alumina, hydrochloric acid, hexane, and ethyl acetate (HPLC grade) from Fisher Scientific. Anti-cytochrome P450 IB1 antibody was raised in rat as described (26).

Microsome Preparation. Human uterine tissues after excision were immediately transferred to the surgical pathology laboratory, sectioned, placed in ice-cold homogenization buffer (1.14% KCl/10 mM EDTA, pH 7.5), and homogenized with a Tekmar (Cincinnati) Ultra-Turrax homogenizer. Microsomes were prepared by differential centrifugation according to the method of Dignam and Strobel (27). Microsomal pellets were resuspended in storage buffer (0.25 M sucrose/10 mM EDTA, pH 7.5) and frozen in aliquots at −80°C until used. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard (28).

Portions of placentae from healthy women were obtained within 1 hr of term deliveries. The tissues were rinsed repeatedly in ice-cold homogenization buffer to remove excess blood. Microsomes were prepared as described above (27).

Microsome-Mediated CE Formation. A validated direct product isolation assay was used to determine rates of CE formation. The assay method and its validation have been described in detail (29). Briefly, microsomal protein (750–1500 μg), 5 mM NADPH, and 1–100 μM [^{3}H]E_2 as substrate were incubated in 0.1 M Tris·HCl/Hepes buffer (pH 7.4) containing 5 mM ascorbic acid in a final volume of 500 μl at 30°C for 30 min. After termination of reactions by rapid freezing, trace amounts of ^{14}C-labeled CE were added to correct for procedural losses. The CE were then adsorbed onto neutral alumina and washed to remove residual substrate. The CE were eluted from the neutral alumina with 0.25 M HCl and separated by thin-layer chromatography. Blank values, determined with heat-denatured microsomes or by omitting either enzyme or NADPH, were subtracted. Values >125% of blanks were minimum criteria for acceptance. Product formation was proportional to incubation time for up to 20 min and to protein concentrations for up to 3 mg/ml. Incubations with chemical inhibitors or cytochrome P450 antibody were done as described above, except that inhibitors were dissolved in 2 μl of ethanol and the cytochrome P450 antibody was dissolved in water before addition to the incubation mixture.

Statistical significance was determined by one-way ANOVA for 2- and 4-hydroxylase activities after testing for the homogeneity of variances. For the ratio data, the nonparametric

Table 1. Rates of 2- and 4-hydroxylation of E_2 and ratios of 4-OH-E_2/2-OH-E_2 formation by microsomes of human myoma, myometrium, and placenta

	4-OH-E_2, (pmol/mg of protein) per min			2-OH-E_2, (pmol/mg of protein) per min			4-OH-E_2
Patient no.	Myoma	Myometrium	Placenta	Myoma	Myometrium	Placenta	2-OH-E_2
Myoma							
1	0.88	0.22 (4)		0.10	0.04 (2)		8.8
2	0.24	0.03 (8)		0.02	0.01 (2)		12.0
3	0.36	0.12 (3)		0.06	0.04 (1)		6.0
4	0.11	<0.01 (>11)		0.01	<0.01 (>1)		11.0
5	0.52	<0.01 (>50)		0.06	<0.01 (>6)		8.7
6	0.11	<0.01 (>10)		0.03	<0.01 (>3)		3.7
7	0.09	0.03 (3)		0.01	<0.01 (>1)		9.0
8	0.25	0.13 (2)		0.04	0.02 (2)		6.2
9	0.35	ND		0.05	ND		
Mean ± SD	**0.33** ± 0.26*			**0.04** ± 0.03			**7.9** ± 1.4†
Myometrium							
10		0.22			0.08		2.8
11		0.08			0.03		2.7
12		0.04			0.02		2.0
13		0.07			0.05		1.4
14		0.03			0.02		1.5
15		0.03			0.03		1.0
16		0.04			0.03		1.3
Mean ± SD		**0.08** ± 0.08			**0.03** ± 0.02		**2.6** ± 1.7
Placenta							
17			0.03			0.77	<0.1
18			0.07			1.13	<0.1
19			0.02			0.60	<0.1
20			0.08			0.73	<0.1
Mean ± SD			**0.05** ± 0.03			**0.81** ± 0.23	**<0.1**

Incubations were done by using 5 μM E_2. Numbers in parentheses represent fold difference in myoma/surrounding myometrium. ND, not determined (tissues were not available).

*Rates of 4-hydroxylation of E_2 by myoma microsomes are significantly higher than those of 2-hydroxylation and also higher than those of 4- and 2-hydroxylation by myometrial microsomes as determined by one-way ANOVA analysis followed by a contrast analysis ($P < 0.01$).

†Ratios of rates of 4-OH-E_2/2-OH-E_2 formation by myoma microsomes were significantly higher than those by myometrium microsomes, as determined by the Wilcoxon procedure (33.54, $P < 0.001$).

Wilcoxon procedure was used to account for the possibility that the ratios would not have a normal distribution.

RESULTS

Uterine Myoma. The mean rate of 2-hydroxylation of 5 μM E_2 by nine uterine myoma microsomes was 0.04 ± 0.03 pmol/mg of protein per min (Table 1). In contrast, the mean rate of 4-hydroxylation of E_2 was 8-fold higher than that of 2-hydroxylation. A kinetic analysis of CE formation from E_2 by two representative myoma microsomes indicated comparable K_m values (5.9–9.2 and 13.1–17.8 μM, respectively) but significantly higher mean V_{max} values for 4-hydroxylation than for 2-hydroxylation (0.8–1.7 and 0.2–0.3 pmol/mg of protein per min, respectively; $P < 0.05$) (Fig. 1).

α-Naphthoflavone, an inhibitor of cytochrome P450 IA1/IA2 (20), inhibited 2-hydroxylase activity in myoma microsomes by 43% and potently inhibited 4-hydroxylase activity by almost 90% (Table 2). Fadrozole hydrochloride, previously reported to inhibit aromatase (21–23), did not markedly affect 4-hydroxylation at low inhibitor concentrations (2–20 μM), whereas 50 μM concentrations decreased this reaction by 25%. 2-Hydroxylation of E_2 was enhanced from 46% to 21% with 2 μM to 50 μM Fadrozole concentration, respectively (Table 2 and data not shown). Similar effects were obtained with ICI D1033. 1-Ethynylnaphthalene and 2-ethynylnaphthalene, selective inhibitors of cytochrome P450 IB1 (24), did not affect 2-hydroxylation but inhibited 4-hydroxylation by 38% and 52%, respectively. At higher concentrations, these inhibitors inhibited 2-hydroxylation of E_2 by ≈30% and 4-hydroxylation of E_2 by up to 80% (data not shown). 1-Ethynylpyrene and 2-, 3-, and 9-ethynylphenanthrene, known to affect activities of both cytochromes P450 IA1 and IB1 enzymes (24), inhibited E_2 2-hydroxylase activity in myoma microsomes by 29–38% and 4-hydroxylase by up to 89%.

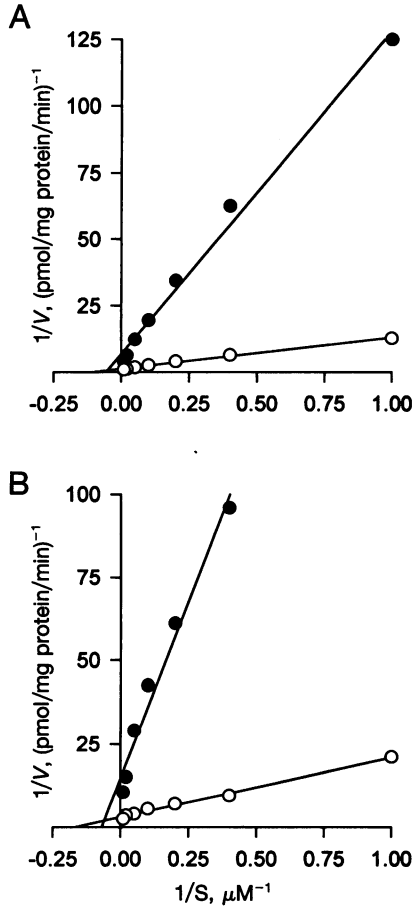

FIG. 1. Double-reciprocal plots of 4-hydroxylation and 2-hydroxylation by two representative myoma microsomes (*A* and *B*). Assays were done as described in text. Each point was the mean of duplicate determinations. Intraassay variations were within 10%. ●, 2-OH-E_2; ○, 4-OH-E_2. (*A*) For 2-OH-E_2 K_m = 13.1 and V_{max} = 0.3; for 4-OH-E_2 K_m = 5.9 and V_{max} = 1.7. (*B*) For 2-OH-E_2 K_m = 17.8 and V_{max} = 0.2; for 4-OH-E_2 K_m = 9.2 and V_{max} = 0.8. *V*, velocity; S, substrate.

A partial but specific inhibition of 4-hydroxylase activity of myoma microsomes was obtained with an antibody raised against cytochrome P450 IB1. At a concentration of 1 mg of cytochrome P450 IB1 antibody per mg of microsomal protein, the rate of 4-hydroxylation of 10 μM E_2 was decreased by 34% from the control value, whereas 2-hydroxylation was not affected (Fig. 2). At an antibody concentration of 2 mg per mg of microsomal protein, the rate of E_2 4-hydroxylation was inhibited by 53%, whereas that of 2-hydroxylation was decreased by 17% (Fig. 2).

In summary, 4-hydroxylation of E_2 is the almost exclusive form of CE formation by microsomes of human myoma. Approximately 50% of this 4-hydroxylase activity is catalyzed by form(s) of cytochrome P450 IB1 recognized by an antibody raised against a rodent form of this enzyme. The small amount of 2-hydroxylase activity in myoma microsomes is affected by specific inhibitors of cytochromes P450 IA enzymes.

Uterine Myometrium. The rates of 2-hydroxylation of 5 μM E_2 by 15 myometrium microsomes were not different from those of myoma microsomes (Table 1). However, mean rates of 4-hydroxylation of 5 μM E_2 by myometrium microsomes (0.08 ± 0.08 pmol/mg of protein per min) were significantly lower than those of myoma microsomes ($P < 0.01$). The lower rates of 4-hydroxylation by myometrium compared to myoma microsomes are also reflected by significantly lower ratios of rates of 4-OH-E_2/2-OH-E_2 formation. Variations were much larger in rates of 4-hydroxylation than 2-hydroxylation. For instance, in two myometrium microsomes (patients 1 and 3), a predominant 4-hydroxylation of E_2 was evident, whereas in one other sample (patient 13), 2- and 4-hydroxylation rates were comparable (Table 1).

A kinetic analysis of 2-hydroxylation of E_2 by three myometrium microsomes revealed that the V_{max} and K_m values (0.1–0.3 pmol per mg of protein per min and 10.1–30.3 μM, respectively) did not differ from values observed with myoma

Table 2. Inhibition of the conversion of E_2 to CE by uterine myoma and myometrium microsomes

Inhibitor	E_2, μM	CE formation as % of control activity	
		2-OH-E_2	4-OH-E_2
Myoma			
ICI D1033	5	131*	90*
Fadrozole hydrochloride	5	146*	93*
α-Naphthoflavone	10	57	12
2-Ethynylphenanthrene	10	66	15
3-Ethynylphenanthrene	10	67	17
9-Ethynylphenanthrene	10	71	28
1-Ethynylnaphthalene	10	97	62
2-Ethynylnaphthalene	10	93	48
1-Ethynylpyrene	10	62	11
Myometrium			
Fadrozole hydrochloride	5	106	85
α-Naphthoflavone	5	43	18
3-Ethynylphenanthrene	5	65†	17†

Incubations were done as described in the absence of inhibitors to determine E_2 2- and 4-hydroxylase activities (controls) and in the presence of 5 μM inhibitor. Values are expressed as percentage of control activities.

*These values were determined at 2 μM inhibitor concentration.

†These values were determined at 50 μM inhibitor concentration.

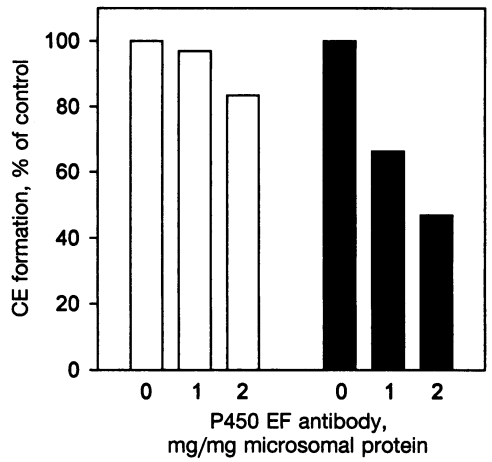

FIG. 2. Inhibition of 4- and 2-hydroxylase activity of human myoma microsomes by an antibody to cytochrome P450 IB1. The assay was done as described in text. Rates of 4-hydroxylation and 2-hydroxylation (black and white bars, respectively) of 10 μM E_2 in the absence of antibody (1.03 and 0.14 pmol/mg of protein per min, respectively) were considered to be 100%. Each point was the mean of duplicate determinations; average intraassay variation was 4.4%. EF, see ref. 33.

microsomes (data not shown). In contrast, V_{max} values for 4-hydroxylation by myometrium microsomes were significantly lower than values for 4-hydroxylation by myoma microsomes (0.5 ± 0.2 and 1.2 ± 0.6, respectively).

As with myoma microsomes, α-naphthoflavone partially inhibited 2-hydroxylation in myometrium (57%) while reducing 4-hydroxylase activity to <20% of controls (Table 2). Fadrozole hydrochloride did not markedly affect the rates of 2- and 4-hydroxylation of E_2 by myometrium microsomes (106 and 85% of control values, respectively, at 5 μM inhibitor concentration (Table 2) and 113 and 85% of control values, respectively, at 50 μM inhibitor concentration; $n = 3$) (data not shown). 1-Ethynylnaphthalene and 2-ethynylnaphthalene inhibited 4-hydroxylation by ≈25% but did not affect 2-hydroxylation (data not shown). 3-Ethynylphenanthrene preferentially inhibited 4-hydroxylation over 2-hydroxylation of E_2.

In summary, human myometrium microsomes predominantly expressed an E_2 4-hydroxylase activity, but the rate of 4-hydroxylation was only ≈20% of that observed with myoma microsomes. 2-Hydroxylation of E_2 did not differ from that in myoma microsomes.

Placenta. Human placenta microsomes converted E_2 almost exclusively to 2-OH-E_2 (Table 1). The mean V_{max} and K_m values for 2-hydroxylation of E_2, calculated from the double-reciprocal plots of four placenta microsomes (data not shown), were 0.98 ± 0.26 pmol/mg of protein per min and 1.4 ± 0.1 μM, respectively. Fadrozole hydrochloride inhibited placental microsome-mediated 2-hydroxylation of 10 μM E_2 by >95% at a 50 μM inhibitor concentration (data not shown). In summary, human placenta microsomes almost exclusively expressed E_2 2-hydroxylase activity. This 2-hydroxylase activity in placenta microsomes is potently inhibited by Fadrozole hydrochloride. Data of CE formation and inhibition are in close agreement with published values (30).

DISCUSSION

Our data identify a specific estrogen 4-hydroxylase activity in human tissues. In uterine myoma, 4-hydroxylation of E_2 is the predominant form of CE formation and occurs at rates 5-fold higher than in surrounding myometrium. Large variations in ratios of 4-OH-E_2/2-OH-E_2 formation seen in uterine myometrium are due to variations in 4-hydroxylase activity, whereas 2-hydroxylase activity varies less and is comparable to that in myoma. This predominance of 4-hydroxylation of E_2 contrasts with CE formation by microsomes of human liver (2, 3, 5) or placenta (Table 1 and ref. 30), which express mainly 2-hydroxylase activity. The conversion of E_2 predominantly to 2-OH-E_2 has been reported to be catalyzed by cytochrome P450 IIIA and aromatase enzymes, respectively (2, 3, 5, 6). In incubations with hepatic or placental enzymes, 4-OH-E_2 is only a minor by-product of the more common formation of 2-OH-E_2.

The enzyme(s) catalyzing this specific 4-hydroxylation of E_2 may be identical or related to cytochrome P450 IB1 {previously identified as EF [for embryo fibroblast (33)]}, a special class of cytochromes P450 previously detected in embryo and uterine endometrial fibroblast cells of mouse and fibroblasts of mammary glands of rats (26, 31–33). This hypothesis is supported by the specific inhibition of ≈50% of 4-hydroxylation of E_2 by an antibody raised against a rodent form of this enzyme. This antibody also inhibits 4-hydroxylation of E_2 in human breast cancer cells such as MCF-7 and T47D, which also express cytochrome P450 IB1 (34). Moreover, inhibition studies by a series of ethynylated hydrocarbon inhibitors suggest that 2-hydroxylation and 4-hydroxylation of E_2 in both myoma and myometrium are catalyzed by distinct enzyme systems.

2-Hydroxylation of E_2 by myoma or myometrium microsomes likely is catalyzed by cytochrome P450 IA and other isozymes. CE formation by aromatase, reported previously for placenta microsomes (6), does not play a role in uterine tissues. This fact has been confirmed here by the inhibition studies using Fadrozole hydrochloride or ICI D1033, which inhibit placental but not uterine conversion of E_2 to catechol metabolites.

The biological role of 4-hydroxylation of E_2 by cytochrome P450 IB1 is unknown. 4-OH-E_2 has been postulated to mediate blastocyst implantation based on physiological studies in the mouse uterus (10). Whether formation of this metabolite is the main function of this form(s) of cytochrome P450 remains to be ascertained. 4-OH-E_2 has also been postulated to participate in E_2-induced tumor formation in hamster kidney (11, 15). This hypothesis is based on the metabolic redox cycling between hydroquinone (CE) and quinone intermediates of CE, including 4-OH-E_2, which may generate potentially mutagenic free radicals (16, 17) and on the decreased dissociation of 4-OH-E_2 from the estrogen receptor complex, which makes this catechol metabolite a long-acting estrogen (18). Consistent with this hypothesis, a specific E_2 4-hydroxylase activity in animals has been identified in hamster kidney (11), mouse uterus (10), and rat pituitary (11), which all serve as animal models of estrogen-induced tumorigenesis. In human tissues, an E_2 4-hydroxylase distinct from the more common 2-hydroxylase has been detected in MCF-7 cells, a breast cancer cell line (4, 34), and in benign and malignant neoplastic mammary tissue (35). In our study, a specific E_2 4-hydroxylase activity has been demonstrated in human uterine myoma and, with lower activity, in surrounding myometrium. The presence of elevated cellular 4-hydroxylase activity in myometrium may predispose to myoma formation. Elevated formation of 4-OH-E_2 in precursor cells could mediate benign tumor formation consistent with a postulated role of this estrogen metabolite in the formation of benign or malignant tumors in rodent models. The postulated development of benign or malignant uterine tumors in women with high E_2 4-hydroxylase activity requires further studies.

In summary, a specific E_2 4-hydroxylase activity has been identified in human uterine myoma and, at lower activity, in uterine myometrium. Elevated 4-OH-E_2 formation in myometrial precursor cells is postulated to mediate benign tumor growth consistent with a role of this estrogen metabolite in estrogen-induced rodent tumors.

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