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REVIEW

Functional role of estrogen metabolism in target cells: review and perspectives

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Cytochrome P450 enzymes that metabolize estrogens are expressed in the mammary gland, uterus, brain and other target tissues for estrogen action, and this results in the formation of hydroxylated estrogens in these tissues. Estradiol metabolites formed in target tissues *at or near* estrogen receptors may either be inactive or have important biological effects, and changes in the activities of estrogen-metabolizing enzymes in target tissues may profoundly influence estrogen action. Although some active estrogen metabolites exert hormonal effects in target tissues by interaction with the classical estrogen receptor, other metabolites appear to elicit unique biological responses that are not associated with activation of this receptor. Therefore, some of the many actions of estradiol may not be caused by estradiol *per se*, but may result from the formation of active estrogen metabolite(s) which function as local mediators or may activate their own unique receptors or effectors. This is an important area in need of more research. The present paper represents a review of the literature and perspectives by the authors on the functional role of estrogen metabolism in target tissues.

Introduction

Estrogens exert diverse biological effects in animals and humans, and many of these effects result from a direct interaction of the estrogen with an intracellular receptor that activates the expression of genes encoding proteins with important biological functions (1–4). One of the most important and notable effects of estrogens is a superpotent mitogenic action in hormone sensitive tissues such as the uterus (5,6) and breast (7–9). Prolonged exposure of target tissues or cells to excessive mitogenic stimulation by natural or synthetic estrogens has long been considered an important etiological factor for the induction of estrogen-associated cancers in experimental animals (10,11) and humans (10,12–18).

Estrogenic hormones are eliminated from the body by metabolic conversion to hormonally inactive (or less active) water-soluble metabolites that are excreted in the urine and/or feces. The metabolic disposition of estrogens includes oxidative metabolism (largely hydroxylations; reviewed in ref. 19) and conjugative metabolism by glucuronidation (20–22), sulfonation (23–27) and/or *O*-methylation (28–30). Members of the cytochrome P450 family are the major enzymes catalyzing β-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH*)-dependent oxidative metabolism of estrogens to multiple hydroxylated metabolites (summarized in Figure 1 and Table I). Although most of the oxidative metabolism of estrogens takes place in liver, some estrogen-metabolizing isoforms of the cytochromes P450 that are usually expressed at low or undetectable levels in liver are *selectively* expressed in certain extrahepatic tissues.

An early suggestion that an estrogen may exert some of its effects because of metabolism in a target tissue came from studies by Fishman and Norton (31). These investigators demonstrated the 2-hydroxylation of estradiol by the rat brain. This work, coupled with earlier studies showing an inhibitory effect of 2-hydroxyestradiol on the inactivation of centrally active catecholamines by catechol-*O*-methyltransferase (32–34), indicated that a locally formed estrogen metabolite may exert a biological effect important for the action of the parent hormone. Although research on the metabolism of estrogens by target tissues has been pursued during the past 20 years, and reviews on certain aspects of this topic have appeared (30,35–40), the functional role or importance of the NADPH-dependent hydroxylations of estradiol and estrone by multiple cytochrome P450 enzymes in target tissues or cells is largely unknown. In this paper, we have reviewed some of our data as well as data by others which collectively suggest a general concept that certain hydroxylated estrogen metabolites formed by specific enzymes in target cells may possess important and unique biological functions that are not directly associated

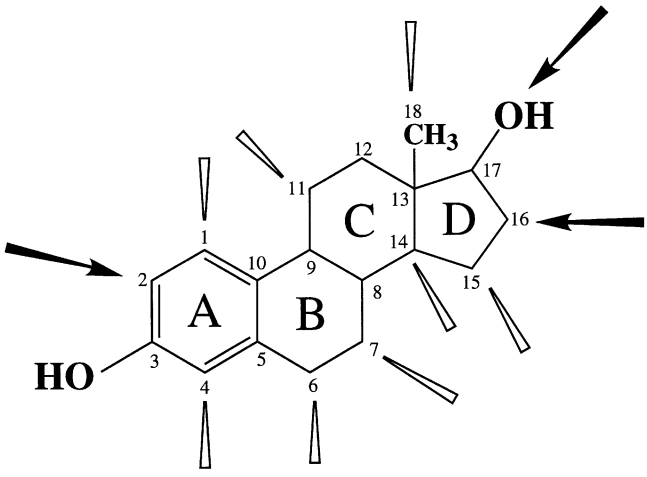

Fig. 1. Sites of oxidative metabolism of estradiol by NADPH-dependent cytochrome P450 enzymes. Major hepatic pathways of estradiol metabolism (2-hydroxylation, 16α-hydroxylation, and estrone formation) are indicated by the solid arrows. Additional information about oxidative estradiol metabolism is summarized in Table I.

***Abbreviations:** NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; COMT, catechol-*O*-methyltransferase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

Table I. Hydroxylated and keto metabolites of 17β-estradiol (estradiol) and estrone. All estrogen metabolites listed were found in biological samples (e.g. tissues, blood, bile, urine) or were formed during *in vitro* incubations of estrogens with enzyme preparations from animals or humans

Positions of oxidation	Estrogen metabolites formed		Major references cited
	Systematic name	Common name	
C-1	1,3,5(10)-Estratrien-1,3-diol-17-one	1-Hydroxyestrone	201
C-2	1,3,5(10)-Estratrien-2,3-diol-17-one	2-Hydroxyestrone	463
	1,3,5(10)-Estratrien-2,3,17β-triol	2-Hydroxyestradiol	52, 464
	1,3,5(10)-Estratrien-2,3,16α,17β-tetrol	2-Hydroxyestriol	465
C-4	1,3,5(10)-Estratrien-3,4-diol-17-one	4-Hydroxyestrone	466, 467
	1,3,5(10)-Estratrien-3,4,17β-triol	4-Hydroxyestradiol	30, 53
	1,3,5(10)-Estratrien-3,4,16α,17β-tetrol	4-Hydroxyestriol	52
C-6	1,3,5(10)-Estratrien-3,6α-diol-17-one	6α-Hydroxyestrone	58, 468
	1,3,5(10)-Estratrien-3,6β-diol-17-one	6β-Hydroxyestrone	193, 468
	1,3,5(10)-Estratrien-3-ol-6,17-dione	6-Ketoestrone	466
	1,3,5(10)-Estratrien-3,6α,17β-triol	6α-Hydroxyestradiol	193
	1,3,5(10)-Estratrien-3,6β,17β-triol	6β-Hydroxyestradiol	193, 469
	1,3,5(10)-Estratrien-3,17β-diol-6-one	6-Ketoestradiol	111, 469
	1,3,5(10)-Estratrien-3,16α,17β-triol-6-one	6-Ketoestriol	111, 470, 471
	1,3,5(10)-Estratrien-3,6α,16α,17β-tetrol	6α-Hydroxyestriol	470
C-7	1,3,5(10)-Estratrien-3,7α-diol-17-one	7α-Hydroxyestrone	47, 471
	1,3,5(10)-Estratrien-3,7β-diol-17-one	7β-Hydroxyestrone	58
	1,3,5(10)-Estratrien-3,7α,17β-triol	7α-Hydroxyestradiol	111, 471
	1,3,5(10)-Estratrien-3,7β,17β-triol	7β-Hydroxyestradiol	58
	1,3,5(10)-Estratrien-3,17β-diol-7-one	7-Ketoestradiol	58
	1,3,5(10)-Estratrien-3,7α,16α,17β-tetrol	7α-Hydroxyestriol	471
C-11	1,3,5(10)-Estratrien-3,11β-diol-17-one	11β-Hydroxyestrone	472
	1,3,5(10)-Estratrien-3-ol-11,17-dione	11-Ketoestrone	472
	1,3,5(10)-Estratrien-3,11β,17β-triol	11β-Hydroxyestradiol	472
	1,3,5(10)-Estratrien-3,17β-diol-11-one	11-Ketoestradiol	472
	1,3,5(10),11-Estratrien-3,17α-diol*	Δ(11)-Dehydroestradiol-17α	473
	1,3,5(10),9,11-Estratrien-3-ol-17-one*	Δ(9,11)-Dehydroestrone	47
C-14	1,3,5(10)-Estratrien-3,14α-diol-17-one	14α-Hydroxyestrone	474, 475
	1,3,5(10)-Estratrien-3,14α,17β-triol	14α-Hydroxyestradiol	111, 474
C-15	1,3,5(10)-Estratrien-3,15α-diol-17-one	15α-Hydroxyestrone	476
	1,3,5(10)-Estratrien-3,15β-diol-17-one	15β-Hydroxyestrone	47
	1,3,5(10)-Estratrien-3,15α,17β-triol	15α-Hydroxyestradiol	201, 111, 470, 477
	1,3,5(10)-Estratrien-3,15α,16α,17β-tetrol	15α-Hydroxyestriol (estetrol)	202, 472
C-16	1,3,5(10)-Estratrien-3,16α-diol-17-one	16α-Hydroxyestrone	478–480
	1,3,5(10)-Estratrien-3,16β-diol-17-one	16β-Hydroxyestrone	481, 482
	1,3,5(10)-Estratrien-3-ol-16,17-dione	16-Ketoestrone	483–485
	1,3,5(10)-Estratrien-3,16α,17β-triol	16α-Hydroxyestradiol (estriol)	483, 486
	1,3,5(10)-Estratrien-3,16β,17β-triol	16-Epiestriol	487–490
	1,3,5(10)-Estratrien-3,17β-diol-16-one	16-Ketoestradiol	480, 491
	1,3,5(10)-Estratrien-3,16β,17α-triol	16,17-Epiestriol	490, 492
C-17	1,3,5(10)-Estratrien-3,17α-diol	17α-Estradiol	493, 494
	1,3,5(10)-Estratrien-3,16α,17α-triol	17-Epiestriol	492
C-18	1,3,5(10)-Estratrien-3,18-diol-17-one	18-Hydroxyestrone	477, 495, 496

*Examples of dehydrogenated estrogen metabolites.

with the parent hormone, but are important for the overall action of the estrogen. Based on a review of data scattered in the literature, we suggest that some of the effects exerted by active estrogen metabolites may be mediated by specific intracellular receptors or effectors which are different from the classical estrogen receptor. In this context, it is noteworthy that a novel estrogen receptor with unknown function(s) has recently been identified in rat prostate and ovary (41) and it is likely that additional isoforms of the classical estrogen receptor also exist (42–45). Studies on possible interactions of the multiple estrogen metabolites with these novel receptors are needed.

In addition to the NADPH-dependent hydroxylation of estrogens catalyzed mainly by cytochrome P450 enzymes, there are also several other pathways of estrogen metabolism in target cells (such as sulfonation, desulfonation, and interconversion between estradiol and estrone) which have been quite well studied in the past. It is known that desulfonation of estrogen sulfates by sulfatase or aromatization of androgens by aromatase in target cells contributes substantially to the formation of *parent* estrogen in these cells and thereby enhances the hormonal stimulation of the classical estrogen receptor. In contrast, metabolism of estrogens by conjugative enzymes (e.g. sulfotransferase) present in target cells may decrease the hormonal activity of estrogens by lowering the intracellular concentration of the parent hormone (25,35,36,39). Although

a major focus of this paper is on NADPH-dependent hydroxylation of estrogens in extrahepatic target tissues, the importance of several other metabolic pathways for the metabolism of estrogens in these target tissues has also been discussed.

Multiple pathways of NADPH-dependent estrogen hydroxylation

Endogenous estrogens (estradiol and estrone) can be hydroxylated at multiple positions (labeled with arrows in Figure 1) by NADPH-dependent cytochrome P450 enzymes. Table I summarizes the presence of *hydroxylated* and *keto* metabolites of estradiol and/or estrone in biological samples (e.g. tissues, blood, urine) or the formation of these metabolites during *in vitro* incubations of estrogens with enzyme preparations from animals or humans. For instance, incubation of [4-^{14}C]estradiol and NADPH with liver microsomes (a crude preparation containing many cytochrome P450 isozymes) from adult male rats resulted in the formation of up to 20 detectable estrogen metabolites (46). Similar formation of multiple estrogen metabolites was observed after incubating [4-^{14}C]-estrone and NADPH with female hamster liver or kidney microsomes (47) or after incubating [4-^{14}C]estrone or [4-^{14}C]estradiol and NADPH with female mouse liver microsomes (48). Since catechol estrogens can undergo metabolic *O*-methylation (30,49), several additional methoxyestrogen metabolites would have been formed if a methylating enzyme system had been included during the incubations.

Several extrahepatic target tissues or cultured cells from target tissues express estrogen-hydroxylating enzyme activities (31,47,50–82). At least nine different isoforms of cytochrome P450 (some are known to metabolize estradiol) have been detected in the mammary gland of the female rat (83), and several isoforms in rat and human breast are subject to developmental and endocrine regulation (83,84). Recent studies showed the presence of high levels of estradiol 4-hydroxylase activity (mediated by cytochrome P450 1B1) in human uterine myoma (80), human breast cancer tissue (81) and a human breast cancer cell line (70,75,85,86). In addition, a unique estradiol 4-hydroxylase activity is expressed in the male Syrian hamster kidney (76,77,79,82), a target organ for estrogen-induced carcinogenesis (87,88). Because of these observations and the strong carcinogenic activity of 4-hydroxyestradiol in a hamster kidney tumor model (88,89), this estrogen metabolite is suspected of playing a role in the development of estrogen-associated cancers in target organs of animals and possibly humans. In contrast to the carcinogenic potential of 4-hydroxyestradiol (88,89), 2-hydroxyestradiol has little or no carcinogenic activity (88,89), and 2-methoxyestradiol (an *O*-methylated product of 2-hydroxyestradiol) exerts potent cytostatic (90–95) and antiangiogenic effects (93,95) which may inhibit the development of estrogen-induced cancers.

Multiple cytochromes P450 are present and are selectively expressed in certain areas of the brain (96–103), but their role for metabolism of estrogens is largely unknown. Enzymes that catalyze the formation of catechol estrogens have been detected in the central nervous system of rats (31,51–53,55–57,66) and the levels of these enzymes in certain regions of the brain are markedly increased (up to 3.5-fold) during lactation (57). It has been postulated that changes in the formation of catechol estrogens in the central nervous system may play a role in gonadotropin release (54,104), ovulation (105,106), and the function of catecholamines (32-34). In addition to the presence of catechol estrogen-forming enzymes in the brain, studies with a partially purified cytochrome P450 fraction from rat brain revealed high catalytic activity for the 6α-hydroxylation of estradiol, and the partially purified enzyme preparation also catalyzed to a lesser extent the 2-, 4-, 6β-, 15α- and 16α-hydroxylations of estradiol (97). The functional role of these hydroxylated estrogen metabolites formed in the brain is not known. It is noteworthy that treatment of rats with ethanol induces the levels of certain cytochromes P450 in the brain (100), but the physiological significance of this effect for the metabolism and action of estrogens in the brain is not known.

Possibly because of very low levels of conjugating enzymes in certain *extrahepatic* target organs, *in situ* metabolism of estrogens may result in accumulation of significant amounts of unconjugated estrogen metabolites in these target tissues. Previous studies showed that very high levels of catechol estrogens are present in the pituitary, hypothalamus, and cerebral cortex (30). High concentrations of 6α-hydroxyestradiol, 16α-hydroxyestradiol (estriol) and 2-methoxyestradiol were present in human follicular fluid specimens, and several additional metabolites were also observed (107).

The observation of multiple pathways for estrogen hydroxylation in liver and, in particular, in estrogen target organs raises the important question of why so many estrogen metabolites are formed? As discussed below, estrogen-metabolizing enzymes in liver and extrahepatic target tissues/cells are under regulatory control by endogenous factors such as sex hormones (83, 108) and by environmental substances such as drugs, pesticides, polycyclic aromatic hydrocarbons, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (70,75,108–111). We believe that many of the multiple estrogen metabolites that are formed in liver or estrogen target tissues may have important but unrecognized biological effects that are necessary for some of the actions of estrogens.

2-Hydroxylation

2-Hydroxylation of estradiol or estrone to a catechol is a major metabolic pathway in the liver whereas 4-hydroxylation to a different catechol represents a quantitatively minor pathway (usually $<$15% of 2-hydroxylation) in this organ (46,77,108,112). Many different isoforms of cytochrome P450 contribute to the 2-hydroxylation of estradiol in the liver. In rats, hepatic 2-hydroxylation of estradiol is catalyzed by cytochromes P450 1A2, 2B1/2B2, 2C6, 2C11, C-M/F (one or more members of the 2D family) and the 3A family (108,111,113–117). In humans, cytochrome P450 1A2 and the 3A family are major enzymes for *hepatic* estrogen 2-hydroxylation (112,118–121). It is of considerable interest that there are large interindividual differences in the 2-hydroxylation of estradiol or estrone by human liver samples (112,118,119) and these inter-individual differences may be reflected by person-to-person differences in estrogen action in different individuals.

NADPH-dependent 2-hydroxylation of estradiol and/or estrone has been observed with microsomes prepared from various extrahepatic tissues such as uterus (64,72,73,80), breast (74,81,122), placenta (50,63,80), kidney (76,79,82), brain (31,51,53,55–57,59) and pituitary (53,66). Catechol estrogen formation by microsomal monooxygenase(s) from human term placenta is predominantly 2-hydroxylation and the 4-hydroxylation is a very minor metabolic pathway in this tissue (50,63,80). Based on studies with selective inhibitors and on

studies using selectively expressed human aromatase (cytochrome P450 19), the 2-hydroxylation of estradiol in human placenta is likely catalyzed by aromatase (123). In contrast to these observations, the 2-hydroxylation of estradiol by MCF-7 human breast cancer cells treated with TCDD appears to be predominantly catalyzed by cytochrome P450 1A1/1A2 (70,75). Since cytochrome P450 3A4 (which has high estradiol 2-hydroxylase activity) is present in several extrahepatic tissues (83,124,125), it is believed that this cytochrome P450 isoform may contribute substantially to estradiol 2-hydroxylation in these tissues.

In addition to the NADPH-dependent 2-hydroxylation of estradiol and estrone, the organic hydroperoxide-dependent peroxidatic pathway may also contribute significantly to the 2- and 4-hydroxylation of these estrogens in liver (126) and estrogen target tissues (63,76,127). This peroxidatic pathway of catechol estrogen formation appears to be catalyzed largely by cytochrome P450 enzymes (40,126). It will be of interest to identify the cytochrome P450 isoforms that have high activity for the peroxidatic formation of catechol estrogens in liver or estrogen target tissues, and to ascertain the physiological significance of this peroxidatic pathway *in vivo*.

Although estradiol and estrone are extensively 2-hydroxylated in liver and extrahepatic tissues, the concentrations of unconjugated 2-hydroxyestradiol and 2-hydroxyestrone metabolites are very low in the systemic circulation (128–130) and in several tissues (131,132), which is probably due to rapid conjugative metabolism (*O*-methylation, glucuronidation, sulfonation, etc.) followed by urinary excretion.

Several important consequences of locally-formed 2-hydroxyestradiol and 2-hydroxyestrone have been suggested: (i) Both catechol estrogens can bind to the classical estrogen receptor, but with a markedly reduced binding affinity (17,30,133,134), and these metabolites possess much weaker hormonal potency as compared with the parent hormone, estradiol (30,135–138). 2-Hydroxyestrone was reported to partially antagonize the growth-stimulatory effect of estradiol in cultured human MCF-7 breast cancer cells (139,140). This growth-inhibitory effect of 2-hydroxyestrogens (at high concentrations) may be due to the interaction of these compounds with the estrogen receptor (139) and/or due to their metabolic redox cycling to generate reactive estrogen quinones and free radicals which are highly cytotoxic (discussed in ref. 141). (ii) By serving as a co-oxidant, 2-hydroxyestradiol strongly stimulates the metabolic cooxidation of arachidonic acid to prostaglandins in the uterus during certain periods of pregnancy (142–145) and thereby may modulate the physiologic effects of arachidonic acid and prostaglandins during pregnancy (146). (iii) 2-Hydroxyestradiol inhibits catechol-*O*-methyltransferase-catalyzed *O*-methylation of catecholamines (32–34), which may exert a modulatory effect on the neurophysiological/pharmacological effects of catecholamines in the central nervous system (147). In addition, catechol estrogens may also modulate intracellular signal transduction (148). It is possible that these modulatory effects of catechol estrogens in the brain may play a role in the feeling of well-being that results from administration of estrogen to ovariectomized or post-menopausal women. (iv) Administration of 2-hydroxyestradiol alters the secretion of prolactin (149,150) and the secretion of luteinizing hormone/follicle-stimulating hormone (LH/FSH) by the anterior pituitary (135). Consistent with a role of locally-formed catechol estrogens in neuroendocrine regulation, estradiol 2- and 4-hydroxylase activity was markedly increased in the anterior pituitary and hypothalamus of female rats during lactation (57). Additional studies suggest that 2-hydroxyestradiol and 2-hydroxyestrone modulate the interaction of the neurotransmitter dopamine with its receptor (151,152), and this may be a mechanism responsible for regulating prolactin secretion (149,150) and other neuroendocrine effects of dopamine. Reports have appeared suggesting the presence of a specific membrane binding site for 2-hydroxyestradiol in the anterior pituitary and endometrial cells, but these observations need to be considered in more detail (153–156). The membrane estrogen binding site in cultured GH3 pituitary tumor cells is suspected of playing a role in causing the rapid release of prolactin (156). (v) 2-Hydroxyestradiol is present in the ovarian follicular fluid of humans (107) and horses (157). An autocrine/paracrine regulatory role for this estrogen metabolite in follicular function has been proposed (158). (vi) 2-Hydroxyestradiol and 2-hydroxyestrone (like 4-hydroxyestradiol) can undergo metabolic redox cycling to generate free radicals such as superoxide and the chemically-reactive estrogen semiquinone/quinone intermediates (159–161) which may damage DNA and other cellular constituents (162–166). Despite their potential for undergoing metabolic redox cycling and generating free radicals, 2-hydroxyestradiol and 2-hydroxyestrone (but not 4-hydroxyestradiol) have little or no tumorigenic activity towards the male Syrian hamster kidney (88,89). Moreover, some studies indicate that treatment of rodents with certain inducers of estradiol 2-hydroxylation may decrease spontaneous tumorigenesis in estrogen-sensitive tissues and this is discussed later.

The lack of carcinogenic activity of 2-hydroxyestradiol and 2-hydroxyestrone (but not 4-hydroxyestradiol) is possibly because the 2-hydroxylated estrogen metabolites, when compared with 4-hydroxyestradiol, have a faster rate of metabolism by catechol-*O*-methyltransferase-catalyzed *O*-methylation (167,168), a more rapid clearance *in vivo* (130,169), and possess weaker hormonal potency in estrogen target tissues (30,134,136–140,170). Moreover, 2-methoxyestradiol (a product of subsequent enzymatic *O*-methylation of 2-hydroxyestradiol) is a very potent inhibitor of tumor cell proliferation (90–95) and angiogenesis (93,95), which may be an important reason for the lack of carcinogenicity of 2-hydroxyestradiol *in vivo*.

4-Hydroxylation

Although 2-hydroxylation of estradiol and estrone is the dominant pathway for catechol estrogen formation in liver microsomes, small amounts of 4-hydroxylated estradiol and estrone are also formed (46,77,82,108,111,112). In rat liver microsomes, 4-hydroxylation of estradiol is catalyzed by cytochromes P450 1A2, 2B1/2, and the 3A family (108,111). In human liver microsomes, the cytochrome P450 3A family is believed to play a major role in the 4-hydroxylation of estradiol (112,121).

In contrast to the above observations indicating that 4-hydroxylation of estrogens is a minor pathway for catechol estrogen formation in liver, recent studies showed that 4-hydroxylation of estradiol is a dominant pathway for catechol estrogen formation in several *extrahepatic* target tissues. For instance, an estradiol 4-hydroxylase activity is expressed in rat pituitary which has little or no activity for 2-hydroxyestradiol formation (66), and an estradiol 4-hydroxylase activity with a low K_m (2–5 μM) was observed in human uterine myometrial and myoma tissues (80). Recent studies indicate that

cytochrome P450 1B1 is an important enzyme for the 4-hydroxylation of estradiol in human breast and uterus (80,86). Expression of the human P450 1B1 gene in *Saccharomyces cerevisiae* produced an enzyme that catalyzed the 4- and 2-hydroxylation of estradiol with K_m values of 0.7–0.8 μM, and turnover numbers of 1.39 and 0.27 nmol product/min/nmol P450, respectively (86). In addition to the NADPH-dependent pathway of catechol estrogen formation, an organic hydroperoxide-dependent peroxidatic pathway may also contribute significantly to the 4- and 2-hydroxylation of estradiol and estrone in liver (126) and estrogen target organs (63,76,127). The different pathways for metabolic formation of 4-hydroxyestradiol by several estrogen target tissues was reviewed earlier by Weisz (38,40).

Selective expression of estradiol 4-hydroxylase activity in target cells does not inactivate the parent estrogen, but may be a mechanism for maintaining strong hormonal activity in these cells or for exerting other unknown biological effects that are not shared with estradiol. As summarized below, several important functions of 4-hydroxyestradiol are already known or have been suggested: (i) 4-Hydroxyestradiol is similar to estradiol in its ability to bind to and activate the classical estrogen receptor (30,133,134,170). Interestingly, the interaction of this estrogen metabolite with the estrogen receptor appears to occur with a reduced dissociation rate compared with estradiol (134,171), suggesting that the association of 4-hydroxyestradiol with the estrogen receptor may last longer than that for its parent hormone, estradiol. (ii) 4-Hydroxyestradiol is hormonally active for stimulating uterine growth when injected into animals (30,170,172), but its uterotropic potency is slightly weaker than that of estradiol (172), possibly due to a faster metabolic clearance than estradiol. It is of considerable interest that addition of 4-hydroxyestradiol to surviving rat or mouse uterine segments *in vitro* was much more effective than estradiol in stimulating the incorporation of [^{14}C]formate into protein (173). Similarly, another study reported that 4-hydroxyestradiol had stronger activity than estradiol in inducing progesterone receptor formation in the rat pituitary under experimental conditions where 4-hydroxyestradiol and estradiol induced a similar level of nuclear estrogen receptor (134). (iii) Administration of 4-hydroxyestradiol (or 2-hydroxyestradiol) alters the secretion of luteinizing hormone/follicle-stimulating hormone (LH/FSH) by the anterior pituitary (135). (iv) 4-Hydroxyestradiol (like estradiol) supports embryo implantation in mice (105,174,175). An increased selective expression of estradiol 4-hydroxylase activity occurs in the pig blastocyst during the preimplantation period (61,65,67,176) and in the uteri of rabbits and mice during embryo implantation (72,73). These observations suggest a possible role of 4-hydroxyestradiol during embryo implantation. (v) Like 2-hydroxyestradiol, 4-hydroxyestradiol also serves as a cooxidant and strongly stimulates the metabolic cooxidation of arachidonic acid to prostaglandins in the uterus during certain periods of pregnancy (142–145). This may modulate the physiological effects of prostaglandins during pregnancy (146). (vi) 4-Hydroxyestradiol inhibits catechol-*O*-methyltransferase-catalyzed *O*-methylation of catecholamines (32–34), which may exert a modulatory effect on the neuropsychologic/pharmacologic effects of catecholamines in the central nervous system (147). (vii) 4-Hydroxyestradiol undergoes metabolic redox cycling (159–161) to generate free radicals such as superoxide and the chemically-reactive estrogen semiquinone/quinone intermediates (shown in Figure 2). These metabolic intermediates may damage DNA and other cellular constituents (162–165), induce cell transformation (166) and initiate tumorigenesis (141,177,178). (viii) 4-Hydroxyestradiol is a strong carcinogen towards the hamster kidney (~100% tumor incidence) under conditions where 2-hydroxyestradiol is not carcinogenic (88,89). The strong carcinogenicity of 4-hydroxyestradiol may be due to its potential genotoxicity (redox cycling plus reactive semiquinone intermediate) as mentioned above and its potent growth stimulatory effect as recently demonstrated with cultured kidney proximal tubule cells (179–181). Although direct injection of estrone-3,4-quinone (derived from 4-hydroxyestrone) into the rat mammary gland did not induce the formation of mammary tumors (182), injection of this reactive estrogen metabolite into 12-day-old male B6/C3/F1 mice four times daily for 4 days did induce hepatomas (178). The ability of 4-hydroxyestradiol or estrone-3,4-quinone to cause kidney or liver tumors in certain animal models is noteworthy, and implicates 4-hydroxylated estrogens as carcinogenic metabolites.

Recent studies suggest that high levels of estradiol 4-hydroxylase activity in estrogen target tissues may play important roles in the development of estradiol-induced tumorigenesis (reviewed in refs 141 and 177). A high level of estradiol 4-hydroxylase activity is expressed in the kidney of male Syrian hamsters (76,77,79,82), the uterus of CD-1 mice (64,73), and the pituitary of rats (66), which are all target organs susceptible to estrogen-induced carcinogenesis (11,87,88,183,184). Interestingly, each of the above three target organs contains very high concentrations of endogenous catecholamines (185) which may provide significant inhibition of catechol-*O*-methyltransferase-catalyzed *O*-methylation of 4- and 2-hydroxyestradiol *in vivo*. Moreover, catechol-*O*-methyltransferase-catalyzed *O*-methylation of 4-hydroxyestradiol is inhibited by 2-hydroxyestradiol, whereas the *O*-methylation of 2-hydroxyestradiol is not inhibited by 4-hydroxyestradiol (168). Therefore, it is likely that 4-hydroxyestradiol will accumulate in these target organs because of inhibition of its *O*-methylation and also because of its rapid formation. 4-Hydroxyestradiol can mediate damage to cellular DNA and other macromolecules according to the mechanism depicted in Figure 2. Moreover, the potent mitogenic effects of estradiol and its hormonally active 4-hydroxylated metabolite formed locally in target cells may stimulate the growth of transformed cells, which is believed to be a necessary component for the full development of estrogen-associated cancers (10,17,179–181).

It is of great interest that human uterine myoma expresses estradiol 4-hydroxylase activity to a greater extent than 2-hydroxylase activity, and the former activity was much higher in tumors than in the ‘normal-appearing’ surrounding myometrium (80). Similarly, elevated estradiol 4-hydroxylase activity has also been observed in human breast cancer tissue compared to normal breast tissue (81). In line with this observation, a previous study reported that 4-hydroxyestradiol appears to be the most abundant estrogen metabolite (4.16 nmol/g tissue) in an extract from a human breast cancer specimen, and several additional metabolites were also observed (186). More studies are needed to confirm the presence of high tissue levels of 4-hydroxyestradiol in human breast cancers. These above interesting results (described in refs 80,81,186), together with studies indicating strong tumorigenic activity for 4-hydroxyestradiol in animals, are consistent with a possible role of metabolically formed 4-hydroxyestradiol in the genesis of estrogen-associated cancers.

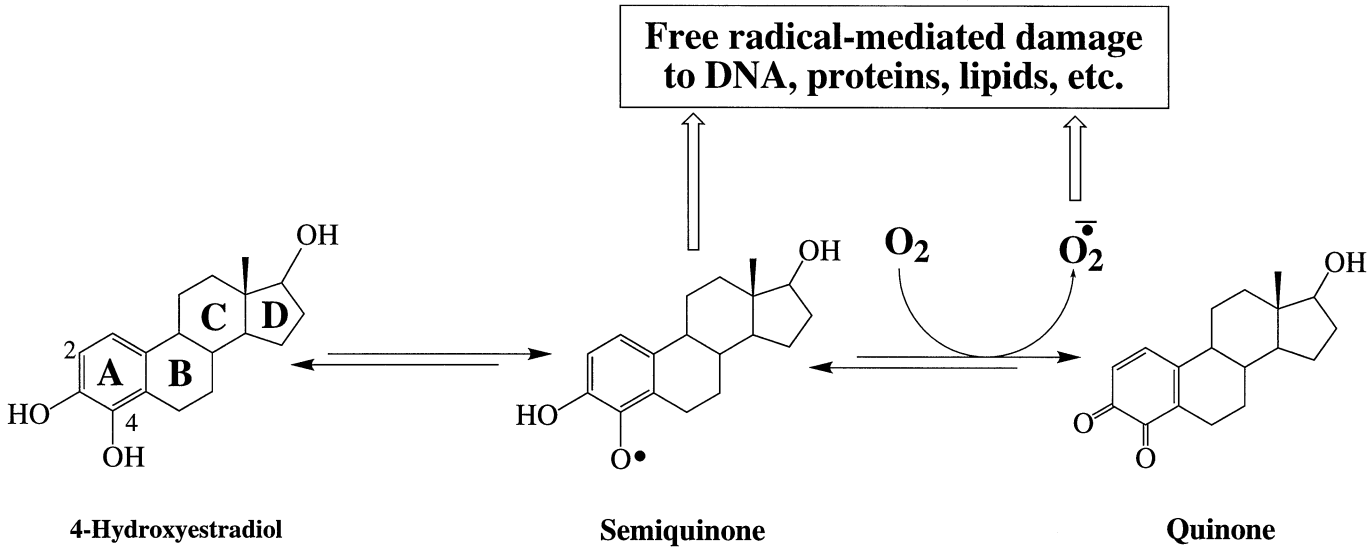

Fig. 2. Metabolic redox cycling of 4-hydroxyestradiol catalyzed by cytochrome P450 enzymes. Although we have only shown the structure of 4-hydroxyestradiol, 2-hydroxyestradiol can also undergo a similar metabolic redox cycling.

Several recent studies have attempted to characterize cytochrome P450 isoform(s) with high estradiol 4-hydroxylase activity in estrogen target tissues, as well as their distribution and regulation. In microsomes from MCF-7 human breast cancer cells treated with TCDD or from human uterine myoma, estradiol 4-hydroxylation is catalyzed predominantly by a newly identified member of the cytochrome P450 1 family, designated as cytochrome P450 1B1 (80,85,86,187). As indicated above, a recent study showed that human cytochrome P450 1B1 isolated from a yeast expression system catalyzes both 4- and 2-hydroxylation of estradiol with low K_m values (<1 μM), but the V_{max} for the 4-hydroxylation of estradiol is ~4-fold higher than the V_{max} for the 2-hydroxylation of estradiol (86).

In mouse or human tissues examined, the mRNA for cytochrome P450 1B1 is expressed in many different tissues or cells (liver, kidney, brain, placenta, breast, uterus, prostate and lymphocytes), and it is also expressed in various steroidogenic tissues such as adrenal gland and ovary (188–190). Cytochrome P450 1B1 mRNA appears to be one of the major cytochrome P450 mRNAs in uterine endometrium and mammary gland (expressed mainly in stromal cells) (187, 188, 190, 191). In contrast, the mRNA for cytochrome P450 1A1 (which catalyzes the 2-hydroxylation of estradiol to a much greater extent than its 4-hydroxylation) is expressed mainly in epithelial cells of mouse uterine endometrium and mammary gland (188,190). The expression of cytochrome P450 1B1 mRNA in several cultured mammary cell lines is not correlated with the presence of the classical estrogen receptor (191).

The expression of cytochrome P450 1B1 mRNA is regulated by multiple endogenous and exogenous factors. In cultured mouse embryo fibroblasts, the expression of cytochrome P450 1B1 mRNA can be regulated by cyclic AMP, adrenocorticotropic hormone (ACTH), TCDD, and polycyclic aromatic hydrocarbons (187,191). The expression of cytochrome P450 1B1-dependent estradiol 4-hydroxylase activity in MCF-7 human breast cancer cells is stimulated by treatment with TCDD (70,85,86), or indolo[3,2-b]carbazole (86), a dietary-derived Ah-receptor ligand (192). However, the expression of cytochrome P450 1B1 mRNA in cultured human primary fibroblasts or JEG-3 chorion carcinoma cells is not stimulated by treatment with 1 pM to 1 μM of TCDD although the expression of P450 1A1 mRNA was markedly induced (189). It will be of considerable interest to characterize the selective expression and differential regulation of cytochrome P450 1B1, and other cytochrome P450 isoforms with estradiol 2- and 4-hydroxylase activity in different cell types in human breast. Such studies may help reveal the functional roles of 2- and 4-hydroxylated estrogens in the physiology and pathophysiology of the human breast.

6- and 7-hydroxylation

The NADPH-dependent 6-hydroxylation of estradiol and estrone by liver from animals and humans has been known for 40 years (193). Recent studies indicate that cytochrome P450 2B1/2B2 in rat liver microsomes is a major enzyme responsible for the 6α- and 6β-hydroxylation of estradiol and that cytochrome P450 1A1/1A2 also catalyzes the hepatic 6α- and 7α-hydroxylation reactions (117). Additional studies showed that treatment of female rats with phenobarbital or dexamethasone markedly stimulated the hepatic 6α- and 6β-hydroxylation of estradiol and that treatment of rats with 3-methylcholanthrene stimulated the hepatic 6α- and 7α-hydroxylation of estradiol (111). It should be noted that cytochromes P450 1A1 and P450 2B1/2 in human liver are very low unless the animals or human subjects are treated with inducers.

The presence of large amounts of 6α-hydroxyestradiol (13.2 ± 0.9 ng/ml, from 11 follicular fluid specimens), in addition to several other estrogen metabolites, was observed in human ovarian follicular fluid (107). 6α- and 7α-hydroxylated metabolites are major biotransformation products of estrone and estradiol in pig uterus (58,78). Pig endometrial cells effectively catalyze the 6α- and 7α-hydroxylation of estrone with low K_m values (<1 μM), and these estrogen-metabolizing enzyme activities were inhibited by cytochrome P450 inhibitors such as ketoconazole (78). 6α-Hydroxylation of estradiol by microsomes from MCF-7 human breast cancer cells is markedly stimulated by exposure of MCF-7 cells to TCDD (70,75), and the addition of anti-rat cytochrome P450 1A1/1A2 IgG to the

incubation mixture inhibited by ~70% the 6α-hydroxylation as well as the 2- and 15α-hydroxylation of estradiol (75). In additional studies, a partially purified cytochrome P450 enzyme from rat brain was shown to catalyze the 6α- and 6β-hydroxylation of estradiol, and this enzyme preparation also catalyzed the formation of 2-hydroxyestradiol, 4-hydroxyestradiol, 6-ketoestradiol, 15α-hydroxyestradiol and 16α-hydroxyestradiol (97). 6- or 7-hydroxylated metabolites of estrone or estradiol do not have appreciable binding affinities for the classical estrogen receptor, and their uterotropic activity is very weak (194,195).

It is noteworthy that a preliminary study by Takagi *et al.* with two chemically synthesized analogs of estrogen-6-sulfates (pyridinium 3-methoxyestra-1,3,5(10)-triene-6α-yl and -6β-yl sulfates) suggest that a highly reactive benzylic carbocation intermediate (at the C-6 position) can be generated in aqueous solution (196), a process which is similar to the formation of chemically reactive intermediates from sulfonated benzylic hydroxyl groups on safrole, estragole or 7,12-dimethylbenz[*a*]anthracene (197–199). Although 6α- and 6β-hydroxylations of estrogens are quantitatively significant pathways in rodents and humans, it is not known whether sulfonation (at the C-6 position) of 6α- or 6β-hydroxylated estradiol or estrone metabolites takes place *in vivo*. If estradiol- or estrone-6-sulfate are formed, they may be highly reactive and genotoxic. Therefore, it will be of interest to determine the relative rate of metabolic formation of 6-sulfonated estrogen metabolites in the body (particularly in estrogen target tissues or cells) and to evaluate the potential genotoxicity or mutagenicity of 6-sulfonated estrogens in these cells.

15α-Hydroxylation

A substantial amount of estrogen 15α-hydroxylase activity is present in certain microorganisms (200), in the human adrenal gland (201,202) and in human fetal liver (203). However, little or no 15α-hydroxylated metabolite was detected during incubations of estradiol with microsomes from non-fetal human liver. In male rats, 15α-hydroxylation is a significant metabolic pathway (46,204) which has been attributed to the male-specific cytochrome P450 2C13 (115,204). It has been reported that male (but not female) rats metabolize estradiol to 15α-hydroxyestrone and 15α-hydroxyestradiol that are excreted into the bile *in vivo* (204). A recent study from our laboratory showed that treatment of immature or adult female rats with 3-methylcholanthrene increased hepatic microsomal estradiol 15α-hydroxylase activity by 5- to 16-fold and cytochrome P450 1A1 was the predominant isoform responsible for the increased 15α-hydroxylation reaction (111).

15α-Hydroxyestrone is present in large amounts in human feces during pregnancy (205). Several earlier studies suggested that the fetal-placental unit is a principal producer of 15α-hydroxylated estrogens (206–209). In the late stages of human pregnancy, urinary 15α-hydroxyestriol (15α,16α-dihydroxyestradiol; estetrol) is excreted in amounts that exceed those of all other estrogens except 16α-hydroxyestradiol (estriol) and 16α-hydroxyestrone (210–214). Large amounts of estetrol have been reported to originate from fetal liver at the late stages of pregnancy, and high levels of this metabolite are found in the serum of infants shortly after birth (215). Although the physiological role of estetrol is unknown, some studies suggested that the amount of estetrol excreted in the urine by the expectant mother can be used as an indicator of fetal well-being (211,213,214,216,217).

On days 12 and 13 of pregnancy in pigs, peri-implantation blastocysts selectively expressed estradiol 15α-hydroxylase activity at 15- to 70-fold higher levels than were expressed during other days of pregnancy (218). The biological significance of this remarkable and short-lived increase of estradiol 15α-hydroxylase activity is not known. Since the 15α-hydroxylation of estradiol results in metabolites that have $<$1% of estradiol's binding affinity for the classical estrogen receptor and little or no estrogenic activity (170,219), perhaps the rapid metabolic conversion of estradiol to 15α-hydroxyestradiol and estetrol protects certain target cells of the embryo and developing infant from exposure to very high concentrations of endogenous estrogenic hormones. Alternatively, these 15α-hydroxylated estrogens may exert other important yet still unrecognized effects. Further research is needed to determine the physiological function(s) of 15α-hydroxyestradiol and estetrol.

16α-Hydroxylation

In male and female rats, the constitutively expressed hepatic cytochrome $P450_{C-M/F}$ (thought to be a member of the 2D family; see ref. 116) has high activity for the 2- and 16α-hydroxylation of estradiol and weak activity for 6α-, 6β- and 15α-hydroxylation of estradiol and for the conversion of estradiol to estrone (114). Cytochrome P450 2C11, a male specific isoform in rats, also catalyzes estrogen 16α-hydroxylation (115) and liver microsomes from male rats catalyze the 16α-hydroxylation of estradiol to a much greater extent than liver microsomes from female rats (46). These results suggest that the male specific cytochrome P450 2C11 may have an important role in the 16α-hydroxylation of estrogens. Studies with prototype liver microsomal enzyme inducers indicate little or no induction of the 16α-hydroxylation of estradiol in female rats treated with phenobarbital, 3-methylcholanthrene, dexamethasone, isoniazid or clofibrate (111). A preliminary report by Blume *et al.* suggests that a new member of the cytochrome P450 2D family may have an important role in the 16α-hydroxylation of estrogens in mouse mammary cells (220). In humans, a recent study showed that cytochrome P450 3A4 has strong catalytic activity for estrone 16α-hydroxylation (119).

16α-Hydroxylated estrogen metabolites were found to possess some unique properties: (i) 16α-hydroxyestrone and 16α-hydroxyestradiol, like 4-hydroxyestradiol, retain potent hormonal activity by activating the classical estrogen receptor (221). (ii) A covalent reaction of 16α-hydroxyestrone with the estrogen receptor has been reported (222), and there is a preliminary study suggesting that 16α-hydroxyestrone may activate classical estrogen receptor-mediated oncogene expression and growth stimulation for a prolonged period (223). Mechanistically, a Schiff base is formed from 16α-hydroxyestrone by reacting with amino groups in proteins (illustrated in Figure 3; ref. 224). In principle, 16α-hydroxyestrone may also react covalently with other amino-containing macromolecules (e.g. DNA), but this has not been described.

Bradlow, Fishman and their colleagues (reviewed in refs 225–227) have suggested that increased formation of 16α-hydroxyestrogen metabolites may be associated with an increased risk for developing mammary cancer in mice and humans based on the following observations. (i) By using [16α-^{3}H]estradiol and measuring ^{3}H release into body water, these investigators reported that whole-body 16α-hydroxylation of estradiol was ~50% greater in post-meno-

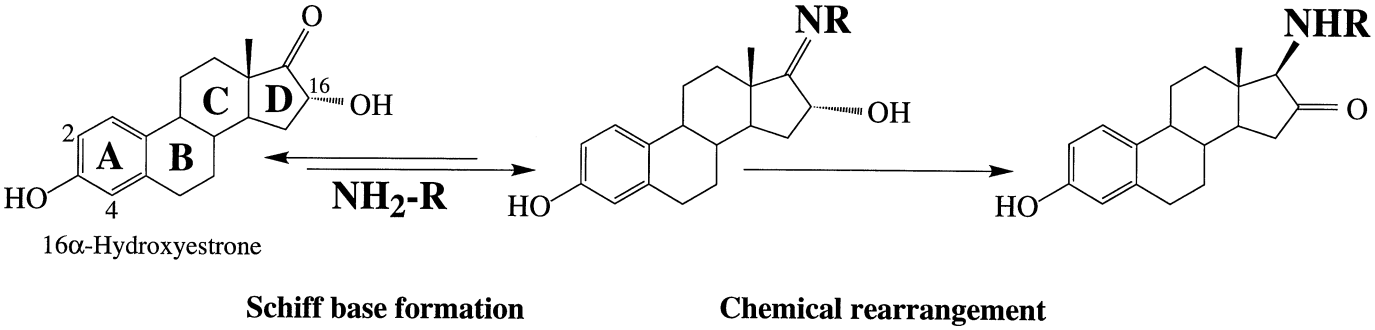

Fig. 3. Formation of stable adducts of 16α-hydroxyestrone with amino-containing macromolecules. The reversible reaction between 16α-hydroxyestrone and the amino group results in the formation of a Schiff base, which will further form a stable 16-keto-17β-amino estrogen adduct via a Heyns rearrangement.

pausal patients with breast cancer than in healthy control subjects and enhanced 16α-hydroxylation was also detected in healthy women at high risk for breast cancer (from cancer-prone families) (228,229). (ii) Estrogen 16α-hydroxylation was higher in terminal duct lobular units in cancerous or non-cancerous tissues from women with breast cancer compared with breast tissue (reduction mammoplasties) from women without cancer (74). Treatment of cultured human mammary tissue with 7,12-dimethylbenz[*a*]anthracene (DMBA) coordinately increased the ras protooncogene expression and estradiol 16α-hydroxylation in terminal duct lobular units (230). (iii) 16α-Hydroxyestrone increased unscheduled DNA synthesis and anchorage-independent growth of mouse mammary epithelial cells in culture, which suggests possible genotoxicity for this estrogen metabolite (231). In addition, 16α-hydroxyestrone (but not 2-hydroxyestrone) enhanced the carcinogen-initiated growth stimulation of cultured mouse mammary epithelial cells (231,232). (iv) Animal studies showed that in several different strains of mice with varying incidence of spontaneous mammary tumors, the extent of 16α-hydroxylation of estradiol was positively correlated with their mammary tumor incidence (233). (v) Treatment of C3H/OuJ mice with indole-3-carbinol, a compound derived from vegetables of the Brassica family (such as broccoli and brussel sprouts) and a precursor of a potent Ah-receptor ligand, indolo[3,2-b]carbazole (192), increased the hepatic 2-hydroxylation but not the 16α-hydroxylation of estradiol (234–236). This treatment of mice or rats with indole-3-carbinol was associated with a decreased incidence of spontaneous mammary or endometrial cancer (237,238). (vi) Certain pesticides and polychlorinated biphenyls, which have been postulated to increase the risk of human breast cancer (239,240), were reported to increase the rate of 16α-hydroxylation of estrone and decrease the rate of 2-hydroxylation of estrone (241) in MCF-7 cells, a human breast cancer cell line.

Although the data described above suggest that increased formation of 16α-hydroxylated estrogen metabolites (relative to the formation of 2-hydroxylated estrogen metabolites) may be associated with an elevated risk of breast cancer, it should be noted that these studies have not yet received sufficient confirmation by other investigators using different experimental settings. One concern is the questionable validity of the ^{3}H release assay used in the above studies which determines the release of tritium from 2-^{3}H- or 16α-^{3}H-labeled estrogens to measure 2- and 16α-hydroxylation *in vivo* and, in particular, to measure the very low levels of estrogen 2- and 16α-hydroxylase activity in uninduced mammary explants or cells *in vitro* (usually <1 pmol/mg microsomal protein/min). It should be noted that a substantial amount of non-enzymatic release of tritium from [2-^{3}H]estradiol has been noted earlier in the tritium release assay for estradiol 2-hydroxylation (55,56,59). A recent study has suggested that there indeed exists substantial differences in the absolute rate of *liver* microsomal 2-hydroxylation of estradiol when measured by the tritium release assay vs. a gas chromatography/mass spectrometry (GC/MS)-based product isolation assay (242). In addition, if enolization at the C-16 and C-17 positions is a necessary step for estrogen 16α-hydroxylation as suggested earlier (243), this may also make tritium at the C-16 position labile.

In contrast to the conclusions of Bradlow and his colleagues, a study by Lemon *et al.* suggested that increased urinary excretion of catechol estrogens but not 16α-hydroxylated estrogens is correlated with an increased risk for non-familial breast cancer (244). More recent studies by Aldercreutz and his colleagues showed that a Finnish population (with high risk for developing breast cancer) had an increased urinary excretion of catechol estrogens relative to 16α-hydroxylated estrogens when compared with an Oriental population at a lower risk (245,246). These investigators suggested that the main risk factor for the Finnish women may be related to high estrogen levels and greater estrogen production than occurred in the Oriental women.

Finally, several additional questions concerning the possible etiological role of 16α-hydroxylated estrogens in hormonal cancer still need to be addressed: (i) 16α-hydroxyestrone and 16α-hydroxyestradiol (estriol) are only very weak carcinogens in the estrogen-induced hamster kidney tumor model under experimental conditions that produced a 100% tumor incidence in animals treated with estradiol or 4-hydroxyestradiol (88,89,180). The question of whether the lack of substantial carcinogenicity for 16α-hydroxyestrone and 16α-hydroxyestradiol (estriol) observed in this tumor model also occurs in other animal tumor models or humans is not known. (ii) Although pregnant women produce very large amounts of 16α-hydroxyestradiol (estriol) and 16α-hydroxyestrone during normal pregnancy (247,248), full term pregnancy does not increase their breast cancer risk, but actually decreases their risk of breast cancer (249–251). (iii) Although 16α-hydroxyestrone is capable of interacting covalently with amino groups in proteins (as depicted in Figure 3), there is not sufficient evidence to indicate *in vivo* genotoxicity or strong carcinogenicity for the 16α-hydroxylated estrogens. In view of these concerns, we conclude that the evidence is not sufficiently strong to support the hypothesis of an etiological role of 16α-hydroxylated estrogens in cancer development.

In summary, we have described the 2-, 4-, 6α-, 6β-, 7α-, 15α- and 16α-hydroxylation of estradiol or estrone by cytochrome P450 enzymes present in liver and, in particular, in estrogen target tissues or cells. Many other hydroxylated metabolites of estradiol and estrone (not discussed here, but summarized in Table I) have been identified during *in vitro* incubation of an estrogen with enzyme preparations from animals or humans and/or have been identified in urine, blood, or tissue samples obtained during *in vivo* studies. It is not known whether these metabolites are formed in extrahepatic target cells and very little is known about their physiological activities. This is an area in need of more research.

As indicated above, many different isoforms of cytochrome P450 are expressed in estrogen target tissues (e.g. breast, uterus and brain), but the role of these cytochrome P450 enzymes for the metabolism and action of estrogens in estrogen target organs has not received adequate attention. We believe that metabolic conversion of estradiol or estrone to multiple hydroxylated metabolites by cytochrome P450 enzymes in target organs is not only for the inactivation of the hormone, but may also diversify the action of estrogens and provide a mechanism that enables estradiol and estrone to exert unique effects in a tissue/cell-specific manner. It will be of considerable interest to examine each of the hydroxylated estrogen metabolites listed in Table I for their estrogenic activity (mediated by the classical estrogen receptor), as well as for other potentially unique biological effects. A recent study showed that 17-epiestriol (a quantitatively minor metabolite of estradiol) is a potent stimulator of the promoter of the human TGF-β3 gene transfected into cultured cells whereas estradiol displayed little or no effect (252).

It is noteworthy that several recent studies have suggested a role for estrogens in the prevention of Alzhiemer's disease in humans (253–256) possibly by enhancing the plasticity and interactions of brain neurons (257). Multiple cytochromes P450 are present in the brain (96–103), and recent studies have shown that alcohol and other inducing agents stimulate the expression of certain cytochromes P450 in the brain (100). These effects of environmental chemicals may modulate estrogen metabolism and action in the central nervous system. It will be of interest to determine whether the reported beneficial effects of estrogen in the central nervous system depend on or are modulated by the metabolism of the hormone by specific isoforms of cytochrome P450 in the brain.

Modulation of estrogen metabolism and action by inducers of estrogen hydroxylation

In earlier studies, we pointed out that steroid hormones are metabolized by the same monooxygenases (cytochrome P450 enzymes) that metabolize drugs and other xenobiotics (258,259). Accordingly, factors that influence the metabolism of xenobiotics also influence the metabolism of steroid hormones (258,259). Cytochrome P450-dependent xenobiotic- and steroid-metabolizing enzymes in liver are regulated by genetic and environmental factors (108–110, 260–267). In studies started over 30 years ago, we found that treatment of rats with phenobarbital or other drugs or with certain halogenated hydrocarbon insecticides increased liver microsomal monooxygenase activity for the metabolism of estradiol, estrone, progesterone, and testosterone (reviewed in refs 109,110,260). The stimulatory effect of liver microsomal enzyme inducers on steroid metabolism was paralleled *in vivo* by a decreased action of the steroids. Pretreatment of rats with phenobarbital or several other enzyme inducers decreased (i) the uterotropic effects of estradiol, estrone and certain oral contraceptive steroids (268–271), (ii) the anesthetic effects of progesterone and deoxycorticosterone (272,273), and (iii) the growth-promoting effects of testosterone on the seminal vesicles (274,275). In contrast to these results, inhibition of hepatic cytochrome P450 enzymes by treatment of rats with carbon tetrachloride enhanced the action of estradiol on the uterus (276).

Additional studies from our laboratory showed that treatment of adult female rats with phenobarbital stimulated the liver microsomal metabolism of estradiol to 2-, 4-, 6α-, 6β- and 14α-hydroxyestradiol whereas treatment with 3-methylcholanthrene stimulated the 6α-, 7α- and 15α-hydroxylation of estradiol (111). Treatment of female rats with dexamethasone stimulated the hepatic microsomal enzyme activities for the 2-, 4-, 6β-, 7α- and 14α-hydroxylation of estradiol and the formation of several nonpolar unidentified metabolites of estradiol (111). Altogether, at least a dozen estradiol metabolites were formed by liver microsomes from female rats treated with different inducing agents (111).

Studies in humans showed that cigarette smoking (exposure to polycyclic aromatic hydrocarbons and other inducers) or treatment of people with phenobarbital and other anticonvulsant drugs stimulated the metabolism of xenobiotics and steroid hormones (reviewed in refs 110 and 260). Female smokers have enhanced 2-hydroxylation of estradiol (277,278), and lower serum and urinary levels of estradiol and estrone (279–281) which helps explain why these individuals have a higher risk for osteoporosis (282–284) and a lower risk for endometrial cancer (282,284,285). Women taking phenobarbital and other anticonvulsants have an increased risk of osteoporosis which was thought to result from enhanced metabolic inactivation of 25-hydroxyvitamin D_3 (286–288), but enhanced metabolism of estrogen to inactive metabolites may also play a role in the increased risk of osteoporosis seen in women treated with anticonvulsants. More research is needed to evaluate this possibility. It will also be of interest to determine whether women taking phenobarbital and other anticonvulsant drugs that enhance estrogen metabolism have a decreased risk of breast or endometrial cancer.

Studies by Bradlow and his colleagues showed that chronic administration of indole-3-carbinol stimulates the 2-hydroxylation of estradiol (234–237) and inhibits mammary preneoplasia and spontaneous mammary tumors in female C3H/OuJ mice (234,237). An inhibitory effect of indole-3-carbinol on the formation of spontaneous endometrial cancer was also observed in rats (238). In addition, Bradlow and his colleagues reported a stimulatory effect of indole-3-carbinol on the 2-hydroxylation of estradiol in humans (289,290) and suggested that indole-3-carbinol may be an effective chemopreventive agent for breast cancer in women (226,229). However, the use of indole-3-carbinol and other Ah receptor agonists as chemopreventive agents against estrogen-dependent human cancers through induction of estrogen 2-hydroxylation should receive more careful evaluation because: (i) Some studies suggest that total catechol estrogen production is positively associated with an increased risk of breast cancer in women (244–246). (ii) Indole-3-carbinol, which is converted to a potent Ah-receptor agonist, indolo[3,2-b]carbazole, in the acidic stomach (192), and other Ah-receptor agonists (such as TCDD) have been reported to increase the rates of estrogen

4- and 2-hydroxylation in liver and estrogen target cells (70,75,86,235). For instance, treatment of rats with indole-3-carbinol resulted in increased rates of hepatic 4- and 2-hydroxylation of estradiol by 4- and 2-fold, respectively (235), and treatment of cultured MCF-7 breast cancer cells with indolo-3-carbazole or TCDD increased the rates of 4- and 2-hydroxylation of estradiol by >10-fold (70,75,86). The stimulatory effect of indole-3-carbinol on estradiol 4-hydroxylation has not been carefully considered in mammary cancer chemoprevention studies in animal models and humans (226,289,290). A chemopreventive strategy for human breast cancer by employing Ah receptor agonists that also enhance the 4-hydroxylation of estrogens to potential carcinogens should receive more attention prior to extensive clinical trials, although indole-3-carbinol treatment may turn out to be an effective modality for inhibition of estrogen-related carcinogenesis and studies in this area should be encouraged.

Recent studies from our laboratory indicated that chronic administration of sodium phenobarbital (0.05% in the drinking water) for 16 months very strongly inhibited the formation of spontaneous mammary tumors in female C3H/OuJ mice. This effect was accompanied by a several-fold increase in the liver microsomal 2-hydroxylation of estradiol with little or no change in estradiol 4- and 16α-hydroxylation (B.T.Zhu and A.H.Conney, unpublished data). It was observed in this study, however, that sodium phenobarbital administration increased the formation of liver tumors. It will be of interest to do additional dose–response studies to determine whether lower doses of sodium phenobarbital will inhibit mammary carcinogenesis without enhancing liver tumor formation. It is important to note that epidemiology studies have not found an enhanced risk of liver cancer in people treated with phenobarbital and other anticonvulsants.

Increased hepatic estradiol metabolism should result in decreased circulating levels of estradiol as well as decreased hormonal activity (269,291), but selective modulation of estrogen metabolism in target tissues or cells should not be expected to affect the blood and urinary levels of estrogens and their metabolites because of the very low activity of estrogen-metabolizing enzymes in target tissues relative to the liver. Previous studies showed that treatment of animals with TCDD (an environmental chemical with potent monooxygenase inducing activity) resulted in a marked antiestrogenic effect (292–294), but the blood level of estradiol was not significantly influenced (294). Additional studies revealed a marked stimulatory effect of TCDD on the 2-, 4-, 6α- and 15α-hydroxylation of estradiol in cultured human MCF-7 breast cancer cells (70). The stimulatory effect of TCDD on oxidative estradiol metabolism in cultured MCF-7 cells was associated with an inhibitory effect on estradiol-induced formation of transformed foci and plasminogen activator activity (295,296). Other studies indicated that administration of TCDD to mice injected with MCF-7 human breast cancer cells inhibited the growth of these explants (296), presumably by enhancing estrogen metabolism in the breast cancer cell explant and/or in host tissues. These results indicate that Ah receptor agonists such as TCDD can exert multiple effects on estrogen action in target cells due to altered estrogen metabolism. These effects include decreased estrogenic hormonal stimulation due to lower levels of estradiol, increased exposure to 2-hydroxyestradiol or 2-methoxyestradiol (potentially protective metabolites), and an increased exposure to 4-hydroxyestradiol (a genotoxic/carcinogenic metabolite of estradiol) (70). The *net* biological effect of TCDD in different target tissues/cells may differ depending on the relative amounts of the different metabolites formed.

It is of considerable interest that although prolonged administration of TCDD to female rats enhanced the incidence of liver tumors, this treatment inhibited the formation of spontaneous tumors in the uterus, mammary gland, and pituitary (297), which are all target tissues for estrogen action. Whether the inhibitory effect of TCDD on spontaneous tumor formation in estrogen target tissues is attributable to its strong stimulatory effect on estrogen metabolic *inactivation* in these target tissues is worthy of further investigation. Careful dose–response studies with TCDD are needed to determine whether the beneficial or toxic effects predominate at low dose levels of TCDD.

Enzyme-catalyzed interconversion between estrone and estradiol

17β-Hydroxysteroid dehydrogenase (17β-HSD) is a group of intracellular isozymes catalyzing interconversions between estradiol and estrone (298). These enzymes are widely distributed in human tissues, not only in classical steroidogenic tissues, such as placenta (299–301) and ovary (302), but also in a large number of peripheral intracrine tissues (303), including adipose tissue (304), skin and vaginal mucosa (305), endometrium (306), breast and cultured mammary cancer cells (307–313), red blood cells (314), and liver (315). Interconversion between estradiol and the less active hormone, estrone, by 17β-HSD in target cells has long been recognized as an important regulatory mechanism for modulation of estrogen action in these cells (84,298).

Although 17β-HSD activity has been detected in several target tissues, many early studies focused on the functional role of this enzyme in the uterine endometrium. 17β-HSD activity is localized in the secretory glandular epithelium and proliferative endometrium of the uterus (316), and this enzyme activity is increased >10-fold in the uterus during the secretory phase of the menstrual cycle compared to that during the proliferative phase (317). In humans as well as in all other mammalian species examined, this increase of uterine 17β-HSD during the secretory phase is mediated by increased concentrations of progesterone (310,317,318). Previous studies showed that uterine 17β-HSD predominantly catalyzes the conversion of estradiol to estrone using NAD (and to a lesser extent, NADP) as a cofactor, resulting in significant loss of estrogenic hormonal stimulation in the uterus (298,315). It is noteworthy that progesterone is included in many estrogen preparations (e.g. post-menopausal estrogen supplements and birth control pills) since it reduces the risk of uterine endometrial cancer in women taking estrogens (18). A major mechanism for the beneficial effects of progesterone in preventing uterine endometrial carcinoma may be attributed to its induction of uterine 17β-HSD, as well as estrogen sulfotransferase (315,317–319).

It is of considerable interest that 17β-HSD in breast tumors predominantly catalyzes the reductive conversion of estrone to estradiol (308,320), suggesting that breast tumors have different isozymes of 17β-HSD than the uterus which catalyzes the oxidative metabolism of estradiol to estrone (298). Moreover, the reductive activity of 17β-HSD is increased in mammary tumors compared with normal breast that does not contain malignant cells (307,309). This finding suggests that breast tumors may provide themselves with a favorable estrogenic

environment for growth through increasing the metabolic conversion of estrone to estradiol. The 17β-HSD reductive activity appears to be under multifactorial control. Several growth factors and cytokines (IL-1β and IL-6) are among the multiple factors capable of stimulating the expression of this enzyme activity in human breast cancer cells (311,313,321–323).

At least five different isozymes of human 17β-HSD (types I–V) have been isolated and sequenced (320,323–327). They have different substrate and cofactor specificities, tissue distributions, subcellular localizations and catalyze opposite reactions *in vivo*. The diversity of the multiple 17β-HSD isozymes suggests that they have different physiological functions in different target cells (328). For instance, reductive activity in human mammary cells is largely associated with the Type I 17β-HSD that uses NADPH as a cofactor (320), whereas the oxidative activity in uterus is associated with Type II 17β-HSD that mainly uses NAD as a cofactor (298,320). It should be emphasized that selective regulation of the oxidative and/or reductive 17β-HSD activity in estrogen target tissues by chemical intervention or dietary modulation may lead to new approaches for the prevention of estrogen-dependent uterine and mammary cancers in humans.

Finally, it is noteworthy that certain isoforms of cytochrome P450 can also catalyze the NADPH-dependent oxidation of estradiol to estrone (117). Hence, factors that influence the levels of cytochrome P450 enzymes may modulate the action of estradiol both by altering its ring hydroxylation and its conversion to estrone.

Estrogen formation by aromatization of androgens or hydrolysis of estrogen conjugates

Aromatization of androgens

Aromatase (estrogen synthetase; cytochrome P450 19) catalyzes the aromatization of androgens to estrogens and is the rate-limiting enzyme in the biosynthesis of endogenous estrogens (329,330). In addition to endocrine tissues such as ovary and placenta (329,331,332), aromatase activity is found in several non-endocrine tissues such as brain (333–335), adipose tissue (336,337), liver (338), fibroblasts (339,340) and mammary glandular cells (341–343). In adipose tissue, much higher enzyme activity and mRNA levels for aromatase are present in stromal cells than in other cell types (344,345).

The extent of conversion of plasma 4-androstene-3,17-dione to estrone is positively correlated with increased obesity and increased age, suggesting adipose tissue as a significant site of estrogen biosynthesis and as a major source of estrogen in post-menopausal women and in elderly men (346). Unlike estrogen synthesis in the ovary, estrogen production in adipose tissue is not cyclic, but continuous. Although the physiological role of extragonadal estrogen production by the aromatase pathway is not fully understood, several investigators (347–349) have discussed the possible relationship between estrogen biosynthesis in adipose tissue and several disease states, such as endometrial and breast cancer, as well as osteoporosis and chronic amenorrhea in obese women and gynecomastia in obese men.

It is of interest that immunohistochemical staining of human breast tumors for aromatase indicated the presence of aromatase in focal areas of stromal spindle cells but only very low levels were present in tumor epithelial cells, inflammatory cells and normal breast cells (350). Moreover, the aromatase activity is more strongly expressed in adipose stromal cells surrounding mammary carcinoma cells than in stromal cells distal to the tumor (344,345), suggesting enzymatic aromatization as an important source of estrogen for mammary tumors. Several inhibitors of aromatase have been developed and used in patients with estrogen-dependent tumors with varying degrees of success (351–353).

Recent studies have characterized the human aromatase gene, which consists of 10 exons and spans at least 75 kb (354–358). The aromatase gene contains multiple copies of exon 1, and each copy of exon 1 contains its own promoter for transcriptional activation (356–359). Tissue-specific expression is regulated by alternative use of these multiple promoters (356–360). The expression of aromatase in breast and abdominal adipose tissue is regulated by a promoter that flanks exon 1b whereas exon 1c (with its own separate promoter) is specific for the expression of aromatase in the ovary (356–359). Molecular and epidemiological analyses of tissue-specific utilization of multiple exons 1 and their corresponding promoters revealed a switching from the use of adipose-specific exon 1b to exon 1c in adipose tissue adjacent to carcinomas in most breast cancer patients, whereas aromatase mRNA in adipose tissue distal to the tumor of the same patient was normally transcribed from exon 1b as occurred in the breasts of healthy controls (356–359). A switching from exon 1b to exon 1c was more often observed in breast cancer patients with metastasis than in those without metastasis (361). It is speculated that the switching from the adipose-specific exon 1b to exon 1c may modify the strictly-regulated tissue-specific expression of aromatase thereby leading to over-expression of aromatase in adipose stromal cells adjacent to the tumor.

A variety of factors have been shown to regulate the activity and expression of aromatase in cultured adipose stromal cells. Examples of these regulatory substances include glucocorticoids, cAMP analogs, phorbol esters and a variety of growth factors such as epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, transforming growth factors α and β, and tumor necrosis factor (360). It will be of interest to identify factors that govern the switching from the use of adipose-specific exon 1b to the use of ovary-specific exon 1c in transcriptional activation of aromatase synthesis in adipose stromal cells near mammary tumors.

Formation and hydrolysis of estrogen sulfates and glucuronides

It has been well documented that metabolic conjugation of estradiol and estrone to glucuronides and sulfates by conjugating enzymes in liver and target cells decreases their hormonal activity by facilitating their excretion (25,35,36,39). However, it has also been known for years that dehydroepiandrosterone (DHEA)-sulfate, a sulfonated steroid with a very slow metabolic clearance rate (<1% of DHEA; ref. 362), is an important intermediate precursor for the biosynthesis of several steroidal hormones in many different types of cells. The concept that conjugated estrogens (formed mainly in liver) may also be important precursors for metabolic formation of hormonally active estrogens in target cells has received ever-increasing attention and support (363–370).

In post-menopausal women, the plasma level of estradiol is very low, but its concentration in uterine endometrium and breast cancer tissues can be 10- to 50-fold higher than the circulating estradiol concentration (371,372). The concentrations of estradiol in the uterine endometrium and breast cancer tissue obtained from post-menopausal women are similar to

those observed in premenopausal women (365,369,371,372). It is believed that estrogen biosynthesis in extragonadal tissues and, in particular, in estrogen target organs may contribute significantly to the unexpectedly high tissue levels of unmetabolized estrogens in post-menopausal women. Since estrone-3-sulfate is a major circulating estrogen metabolite with an overall low clearance rate and a long half-life in humans (~9 h) (373,374), estrone-3-sulfate is thought to be an important precursor of active estrogen peripherally in post-menopausal women (37,367,370,375).

Sulfonated estrogens themselves have almost no estrogen receptor binding affinity and their estrogenic activity results from the release of unconjugated estrogens by enzymatic deconjugation in liver as well as in target tissues or cells (e.g. uterine endometrial cells and breast cells) (25, 363–369,376). A recent study in rodents from our laboratory showed that estrone-3-sulfatase in the uterus (a classical target organ for estrogen action) may play a more important role than hepatic sulfatase in mediating the hormonal action of estrone-3-sulfate in this target organ (377). Since estrone-3-sulfate is transported into human breast cancer cells (378) which contain high levels of estrogen sulfatase activity (25,367,368,378), enzymatic hydrolysis of estrone-3-sulfate (together with estrogen synthesis by aromatase in breast tumors) is likely an important source of parent estrogens in breast cancer cells and these pathways of estrogen synthesis are believed to contribute very significantly to the higher concentration of estradiol in breast tumor tissues than in the systemic circulation (37,369,370,372). Several studies have indicated that estrogen formation in human breast tumors in post-menopausal women through desulfonation of estrone-3-sulfate may be more important than the aromatase pathway of estrogen formation by the breast (367,370,375,378). It is noteworthy that a previous study showed that treatment of rats with estrone-3-sulfate significantly stimulated the growth of *N*-methyl-*N*-nitrosourea-induced mammary tumors (379), which is in support of the concept that estrogen sulfates can be effective precursors of estrogenic hormones required for mammary tumor growth *in vivo*.

High levels of estrogen β-glucuronidase, which converts estradiol and estrone glucuronides to their corresponding parent hormones, are expressed in hamster kidney (a target organ for carcinogenesis), and this enzyme activity is stimulated in the kidney following chronic administration of estradiol (380). The high estrogen β-glucuronidase activity in hamster kidney (approximately twice the activity in liver) and the increase of this enzyme activity during chronic estrogen treatment may provide additional amounts of parent estradiol and estrone as well as reactive estrogen metabolites to this target organ and thus may facilitate the development of estradiol-induced kidney tumors (380).

It is of considerable interest that a large amount of β-glucuronidase was found in mammary glandular tissue of female Sprague–Dawley rats at puberty (381), and treatment of these animals with dietary D-glucaro-1,4-lactone (a potent natural inhibitor of β-glucuronidase) strongly inhibited the growth of mammary glandular cells *in vivo* (381). Additional studies also showed that treatment of female Sprague–Dawley rats with β-glucuronidase inhibitors suppressed mammary tumor promotion (an estrogen-dependent process) after initiation with chemical carcinogens (382–384).

It should be noted that estrogen sulfates or glucuronides can also be hydroxylated at multiple positions by NADPH-dependent cytochrome P450 enzymes in liver as well as in extrahepatic target cells (117,385,386). The hydroxylation of conjugated estradiol and estrone in the liver and/or in target cells and further deconjugation (enzymatic hydrolysis) of these metabolites may be an additional source of hydroxylated estrogen metabolites in target tissues or cells.

More research is needed to understand the factors that regulate the expression of estrogen-specific sulfotransferase, UDP-glucuronosyltransferase, sulfatase and β-glucuronidase in estrogen target tissues, as well as to identify genetic and environmental factors that modulate these enzyme activities. The formation of estradiol, estrone and their hydroxylated metabolites by metabolic hydrolysis of estrogen sulfates and glucuronides is a potential target for chemotherapy or chemoprevention of estrogen-associated tumors (e.g. breast and uterine cancers). Developing potent and selective inhibitors of estrogen sulfatase(s) is already an actively-pursued strategy for chemoprevention against mammary tumorigenesis (387–389). We believe that developing potent β-glucuronidase inhibitors with low toxicity and high bioavailability in humans will also be of great potential value for the prevention and treatment of estrogen-associated cancers.

Formation and catabolism of methoxyestrogens and estrogen fatty acid esters

Metabolic hydroxylation and/or conjugation of estrogens make(s) them more water soluble and, at the same time, often markedly reduces or completely eliminates their classical estrogenic activity. However, enzymatic *O*-methylation or esterification with fatty acids leads to the formation of much more lipophilic estrogen metabolites. These metabolites have very long half-lives (390,391) and do not bind to the classical estrogen receptor (392). Recent studies suggest that at least some of these lipophilic estrogen metabolites may have unique biological activities (not associated with the classical estrogen receptor) or they can be further converted to biologically active molecules at or near estrogen receptors.

Methoxyestrogens

The *O*-methylation of catechol estrogens is catalyzed by catechol-*O*-methyltransferase (COMT), an enzyme that also catalyzes the *O*-methylation of physiologically important catecholamines and many other catechols (49,393–395). Catechol-*O*-methyltransferase activity is present in large amounts in liver and kidney, and it also exists in significant amounts in red blood cells, uterine endometrium, the mammary gland and many other tissues (396–399). In all tissues examined thus far, catechol-*O*-methyltransferase activity is found almost exclusively in the cytosol, but some activity is also found in a membrane-bound form (394,400). Because of the rapid enzymatic *O*-methylation of catechol estrogens, 2-methoxyestrone was previously shown to be one of the most abundant estrogen metabolites in human plasma and urine (30,401,402). In pregnant women, the mean plasma concentration of unconjugated 2-methoxyestrone is ~4000 pg/ml (403). Interestingly, 2-methoxyestrone and 2-methoxyestradiol have higher binding affinities for sex hormone-binding globulin than estradiol and 2-hydroxyestradiol (404), and the high binding affinities of these two methoxyestrogens may contribute to their high plasma levels.

The monomethylated estrogen metabolites (structures shown in Figure 4) have little or no estrogen receptor binding affinities (<1%) when compared to estradiol (30,405), and they lack estrogenic effects on the uterus [e.g. lack of effect on uterine

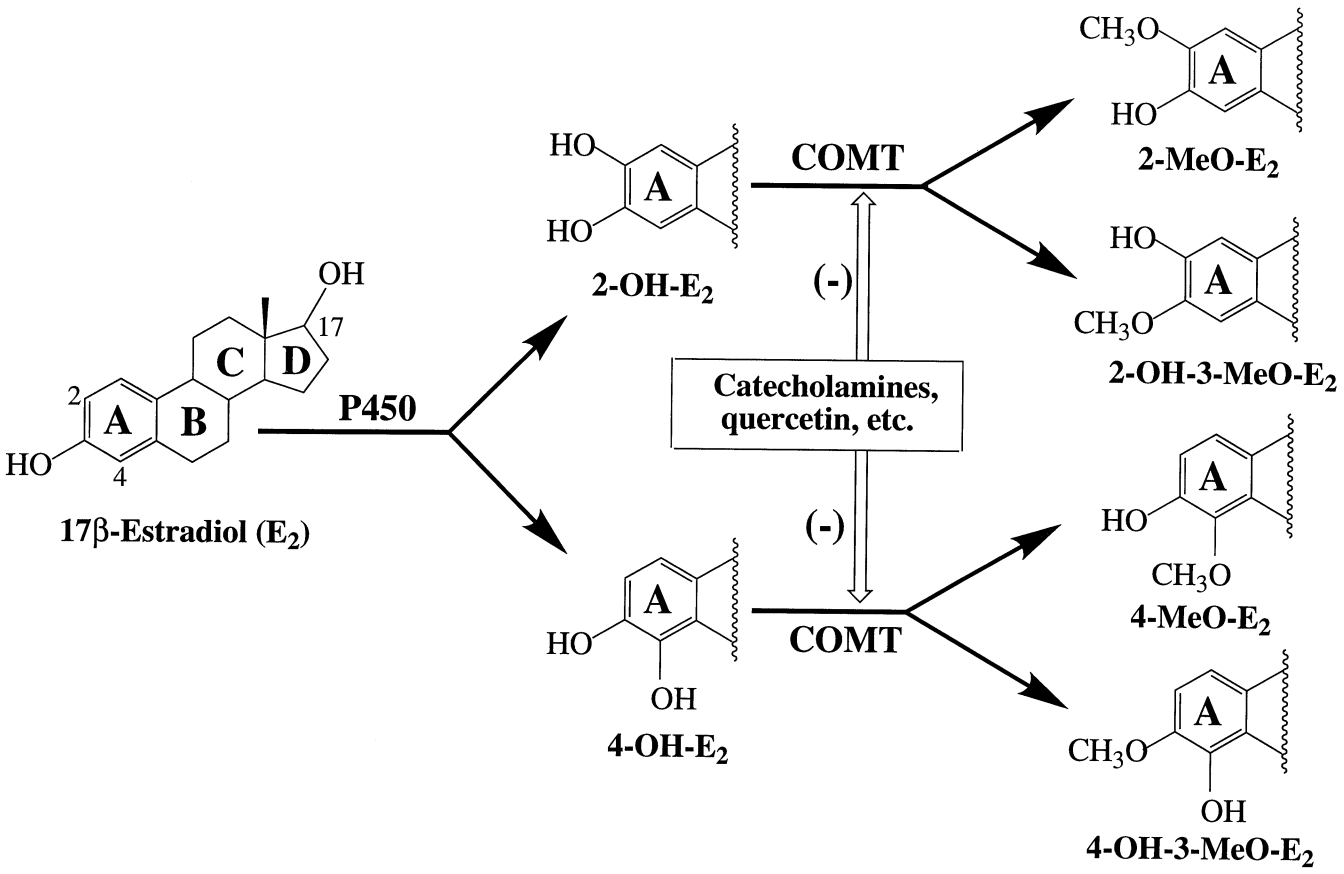

Fig. 4. Structures of monomethylated catechol estradiol metabolites. Estradiol is hydroxylated to form catechol estradiol metabolites (2- and 4-hydroxyestradiol). They can be further *O*-methylated by catechol-*O*-methyltransferase (COMT) to form monomethoxy estradiol metabolites. 2-Methoxyestradiol and 4-methoxyestradiol are the major monomethylated isomers formed from their respective catechol precursors. Although the structures for estrone and its catechol metabolites are not shown, the same metabolic pathways as shown also occur for estrone or its metabolites (a 17-keto group in place of the 17β-hydroxy group of estradiol). Since catechol-*O*-methyltransferase catalyzes the metabolism of a wide spectrum of endogenous and exogenous catechols, the *O*-methylation of catechol estradiol or estrone metabolites is thus subjected to regulation by these catechols (hydroxylated flavonoids, catecholamines, etc.). *Abbreviations used:* 2-OH-E_2, 2-hydroxyestradiol; 4-OH-E_2, 4-hydroxyestradiol; 2-MeO-E_2, 2-methoxyestradiol; 2-OH-3-MeO-E_2, 2-hydroxy-3-methoxyestradiol; 4-MeO-E_2, 4-methoxyestradiol; 4-OH-3-MeO-E_2, 4-hydroxy-3-methoxyestradiol.

wt (221) or peroxidase activity (406)]. Previous studies on the chemical reactivity and potential genotoxicity of catechol estrogens (159–165,178) have led to the suggestion that enzymatic *O*-methylation was primarily a detoxification pathway for these catechol intermediates. However, there are several studies indicating that 2-methoxyestradiol exerts unique biological effects that are not associated with estradiol, 2-hydroxyestradiol or other methoxy derivatives of estradiol (90–94,407–411). For instance, treatment of rats with 2-methoxyestradiol decreases cholesterol and triglyceride levels in the blood and this effect is not associated with activation of the classical estrogen receptor (407–409). In addition, 2-methoxyestradiol inhibits the growth of certain human breast cancer cell lines *in vitro* and *in vivo*, and it is a potential inhibitor of estrogen-dependent carcinogenesis. These studies are discussed below.

2-Methoxyestradiol inhibits the proliferation of several cancer cell lines *in vitro* (90,91,93,95,412), and human breast cancer cell lines (estrogen receptor positive or negative) were particularly sensitive to a cytotoxic effect of 2-methoxyestradiol (412). Additional studies indicated that 2-methoxyestradiol disrupted microtubule function (92,94) and was a potent inhibitor of angiogenesis (93,95). Administration of 2-methoxyestradiol inhibited the growth of transplanted meth-A sarcoma and B16 melanoma in C3H mice (93), and oral administration of 2-methoxyestradiol also inhibited the growth of a human breast carcinoma cell line (estrogen receptor negative) in immunodeficient mice (95). It is noteworthy that 2-methoxyestradiol is among the most potent endogenous inhibitors of angiogenesis known, and its antiangiogenic effect as tested *in vitro* is highly specific and is not shared by several closely related structural analogs (93). The effects of 2-methoxyestradiol to disrupt microtubule function, to inhibit angiogenesis and to inhibit the proliferation of breast cancer cells *in vitro* and *in vivo* suggest that factors enhancing the 2-hydroxylation of estradiol and the subsequent formation of 2-methoxyestradiol may inhibit estrogen-induced breast cancer. It is possible that the stimulatory effect of indole-3-carbinol and phenobarbital on the 2-hydroxylation of estradiol and the subsequent formation of an increased amount of 2-methoxyestradiol can explain the inhibitory effects of indole-3-carbinol and phenobarbital on spontaneous breast carcinogenesis in C3H/OuJ mice.

Recent studies have shown that chronic administration of quercetin (a substrate and inhibitor of catechol-*O*-methyltransferase) to male Syrian hamsters significantly increased the severity of estradiol-induced kidney tumors (413), which was correlated with inhibition of enzymatic *O*-methylation of catechol estrogens during quercetin administration (414). It is of considerable interest that male Syrian hamster kidney,

CD-1 mouse uterus and rat pituitary (tissues that develop estradiol-induced tumors) have very high levels of endogenous catecholamines (up to 50-fold higher than in several non-target tissues in the same animals or in other strains or species) (185). High concentrations of catecholamines in target tissues may inhibit catechol-*O*-methyltransferase-catalyzed *O*-methylation of catechol estrogens (185), which will result in decreased formation of 2-methoxyestradiol and increased tissue concentrations of 2- and 4-hydroxylated estrogens. This effect may enhance tumor formation in these target tissues. Earlier studies in animals showed that stressed mice had an increased incidence of spontaneous breast cancer (415). Interestingly, epidemiological studies have also suggested that sustained stress (associated with an increase in endogenous catecholamine levels) is a risk factor for human breast cancer (416–419), which is consistent with decreased formation of 2-methoxyestradiol together with the possible accumulation of 4-hydroxylated catechol estrogens in mammary tissue.

Since human and rodent breast cancers express catechol estrogen-forming enzyme activity (81,122,127,420,421) and elevated levels of catechol-*O*-methyltransferase (393–395), it is likely that significant amounts of 2-methoxyestradiol are formed in breast cancer cells. Recent studies showed that there are marked person-to-person variations in catechol-*O*-methyltransferase activity in red blood cells (422–424) and liver samples (425), and the distribution of catechol-*O*-methyltransferase activity in the American population appears to follow a polymorphic bimodal pattern (423,425,426). It will be of great interest to compare the risk of breast cancer in people with high or low catechol-*O*-methyltransferase activity. It is expected that individuals with significantly lower COMT activity may have a higher risk of estrogen-associated breast cancer due to decreased formation of antitumorigenic 2-methoxyestradiol and retarded inactivation of catechol estrogen intermediates (particularly 4-hydroxyestradiol which is hormonally active and potentially genotoxic).

The mechanisms by which 2-methoxyestradiol exerts antiangiogenic activity, anti-tubulin activity, and antiproliferative effects on tumor cells lacking the classical estrogen receptor are not known. The high degree of selectivity of 2-methoxyestradiol's antiangiogenic activity (many closely related analogs have poor activity) suggests the possibility of a specific yet unidentified receptor for this action of 2-methoxyestradiol. It will be of great interest to determine the molecular mechanisms of action of 2-methoxyestradiol, to determine whether endogenously formed 2-methoxyestradiol has antitumor effects *in vivo*, and to identify endogenous as well as exogenous factors that regulate the formation and metabolic disposition of 2-methoxyestradiol in target cells. Such studies will help our understanding of the physiological roles of 2-methoxyestradiol, which is a nonpolar estradiol metabolite formed in substantial amounts in many estrogen target cells. These studies may also provide novel mechanism(s) and strategies for inhibiting the formation and growth of hormonal cancers.

Finally, it should be noted that although 4-methoxyestradiol showed little activity in test systems where 2-methoxyestradiol is highly active, whether this methoxyestrogen metabolite has other unique biological functions other than serving as a substrate for 4-hydroxyestradiol formation by demethylation (380) is not known at present.

Estrogen fatty acid esters

In the presence of fatty acid acyl-co-enzyme A, estradiol (at the C-17 position only) can be converted to very lipophilic estrogen fatty acid esters by enzymes present in liver as well as in estrogen target organs such as the uterus, breast and placenta (427–432). In MCF-7 human breast cancer cells, enzyme activity for the formation of estrogen fatty acid esters is highest in the microsomal fraction, but some enzyme activity is also present in the nuclear and mitochondrial fractions (431). Both saturated and unsaturated fatty acid acyl-co-enzyme A derivatives can serve as donors for the esterification of estradiol. It should be noted that although most previous studies have only studied fatty acid ester formation with estradiol as substrate, esterification of 16α-hydroxyestradiol (estriol) has also been shown to occur at the 16α-hydroxy group (432,434).

Estradiol fatty acid esters themselves have little or no estrogen receptor binding affinity (392), and their hormonal activity results from the slow release of the parent estrogen by metabolic cleavage of the fatty acid esters by esterases (430–435). Because of the high lipophilicity of the fatty acid esters of estradiol, these endogenous metabolites are present at very low concentrations in the blood (436–438), but at relatively high concentrations in fatty tissues (432,438). These esters have long half-lives and are extremely potent mammalian estrogens because they serve as a reservoir and direct precursor for estradiol formation (390,391,432,439). Because the mammary gland contains large amounts of fat which can serve as a storage site for fatty acid esters of estradiol and possibly also for fatty acid esters of hydroxylated estrogen metabolites, we suggest that estrogen-fatty acid esters may be particularly important estrogens for the breast ('mammary selective estrogen metabolites').

It will be of considerable interest to advance our knowledge on the formation, storage and, in particular, subsequent hydrolysis of estrogen-fatty acid esters by enzymes selectively expressed in the mammary gland which may be an important pathway for supplying hormonally-active and/or potentially genotoxic estrogens for this target organ. It will also be of interest to study the factors (endogenous or exogenous) that regulate the synthesis and cleavage of estrogen fatty acid esters since factors influencing the formation and hydrolysis of estrogen fatty acid esters in liver and in extrahepatic target cells may profoundly affect the intensity and duration of estrogen action in the body. Recent studies in our laboratory indicated that treatment of rats with clofibrate (a peroxisome-proliferator) stimulates by several-fold the liver microsomal formation of estradiol-fatty acid esters (440). The significance of this effect on estrogen's hormonal action remains to be elucidated.

Estrogen metabolites formed in target cells may have unique functions not associated with classical estrogen receptors: a general hypothesis

Estradiol activates the classical estrogen receptor to exert its major effects and so do some estrogen metabolites that still retain potent estrogenic activity (e.g. 4- or 16α-hydroxyestradiol, and 4- or 16α-hydroxyestrone). Although these four estrogen metabolites as well as some other estrogen metabolites have uterotropic activity in rodents, they are generally less potent than estradiol.

Some estrogen metabolites elicit unique physiological responses with higher potency and activity than the parent hormone estradiol, and some of these unique activities as well as some activities of the parent estrogen cannot be blocked by estrogen receptor antagonists. These results suggest that certain

effects of estradiol and its metabolites are not associated with activation of the classical estrogen receptor. It is becoming increasingly better recognized that certain estrogen metabolites formed in target tissues or cells may serve as local modulators or mediators of specific biochemical events taking place in these target cells. We have indicated earlier in this review that (i) catechol estrogens may modulate the function of dopamine and other catecholamines by inhibiting their enzymatic *O*-methylation and by modulating their interaction with specific receptors; (ii) 4-hydroxyestradiol is carcinogenic and this compound may damage cellular macromolecules such as DNA, proteins and lipids because of metabolic redox cycling to generate reactive intermediates and free radicals; (iii) 2-methoxyestradiol inhibits angiogenesis, the growth of cultured cancer cells and the growth of human mammary cancer cells in immunodeficient mice; and (iv) 17-epiestriol stimulates the promoter of the human TGF-β3 gene transfected into cultured cells whereas estradiol has little or no effect (252). Studies with 17-epiestriol suggest that the interaction of this estradiol metabolite with the classical estrogen receptor is different from the interaction of estradiol with the receptor, and this interaction of 17-epiestriol with the estrogen receptor elicits a new response not shared by estradiol (252). We believe that future studies will identify unique effects of additional estrogen metabolites.

We hypothesize that several active metabolites of estradiol may exert unique effects through interaction with their own specific intracellular receptors or effectors that are refractory to the parent estrogen. Although the functions and structures of these hypothetical intracellular receptors are unknown at present, there are several candidates worthy of consideration. (i) Previous studies showed the presence of multiple variants of the classical estrogen receptor as a result of aberrant splicing of its mRNA (441–444). It will be of interest to determine whether certain active estrogen metabolites may be endogenous ligands for some of these variants. (ii) The nuclear type II estrogen binding site has a low apparent binding affinity for estradiol (445,446) and functionally mediates a growth-inhibitory effect in estrogen-sensitive cells (447). Although previous studies reported methyl *p*-hydroxyphenyllactate as an endogenous ligand for the type II estrogen binding site (448), it is not known whether physiologically active estrogen metabolites are also ligands for the type II binding site. (iii) A novel estrogen receptor expressed in rat prostate and ovary was recently described (41). Estradiol is bound to this receptor with high affinity, and estrone and 16α-hydroxyestradiol were only moderately effective for inhibiting estradiol binding (ligand competition experiments). The binding of other estrogen metabolites to this receptor either as agonists or antagonists have not been determined. It is likely that additional novel isoforms of the classical estrogen receptor may also exist (42–45). (iv) An estrogen binding site in the plasma membrane has been reported (153–156) and this binding site is suspected of playing a role in the rapid release of prolactin in cultured GH3 pituitary tumor cells (156). Although an earlier study suggested that 2-hydroxyestradiol can bind to a membrane receptor site (153), the binding affinity of many other estrogen metabolites towards this membrane receptor is not known. (v) Several DNA sequences that encode proteins with a high degree of structural similarity to members of the steroid receptor superfamily have been identified in animals and humans (449), but their functions and endogenous ligands are not known. These proteins have been called 'orphan receptors'. It will be of considerable interest to determine whether estrogen metabolites with unique functions are endogenous ligands for some of the orphan receptors. (vi) In addition to the candidate receptors mentioned above, other unidentified receptors or specific intracellular effectors (such as transcriptional factors or enzymes) may also be targets for active estrogen metabolites. Whatever the structural identity of intracellular receptors or effectors for active estrogen metabolites, we believe that the interaction of active estrogen metabolites with these specific receptors or effectors in target cells may lead to the expression of new responses or alterations of on-going biochemical processes. This is an area in need of more research.

There are a few examples of biologically active metabolites of certain hormones that have their own receptors. These examples may help our understanding of the hypothesis that certain unique effects of the parent estrogen are mediated by locally-formed metabolites that activate their own specific receptors or effectors in target cells. The pituitary takes up thyroxine (T_4) from plasma and converts it locally into triiodothyronine (T_3), which is the active receptor-binding form of thyroid hormone (450). In addition, there is enhanced conversion of T_4 to T_3 in the cerebral cortex and cerebellum of animals with hypothyroidism (451). The prostate converts testosterone to dihydrotestosterone locally and it is the latter compound that binds to a nuclear receptor with a much higher affinity than testosterone (452). The kidney and gut epithelium (two major target tissues for vitamin D action) selectively express 1α-hydroxylase activity, which converts 25-hydroxyvitamin D_3 (a poorly active prehormone that circulates in blood) to the active hormone, 1α,25-dihydroxyvitamin D_3, in target sites for hormone action (453). These examples suggest that metabolism of hormones in target cells may be a general mechanism for markedly increasing the original hormone's activity and cellular specificity. Moreover, the enhanced enzymatic conversion of T_4 to T_3 in the pituitary of rats with hypothyroidism also exemplifies a metabolic control mechanism by which the body maintains homeostasis by regulating the metabolism of certain hormones in target cells (451). The enzymatic conversion of androgen to estrogen by aromatase is a mechanism that modifies and diversifies the original hormone's action. For instance, the hypothalamic region of the brain in immature rodents converts androgens to estrogens locally by aromatase as a requisite step for male differentiation (334,335). The *in situ* conversion of testosterone to estradiol by aromatase markedly alters the action of testosterone and provides a source of estrogen in these aromatase-containing cells. In this case, metabolically formed estradiol interacts with its own intracellular receptor which is not responsive to its androgen precursor.

By analogy with the above examples, active estrogen metabolites do not need to be present in the systemic circulation in significant quantities to exert their biological effects. The parent hormone estradiol may exert unique functions through metabolic formation of active estrogen metabolite(s) catalyzed by specific metabolizing enzymes that are selectively expressed in estrogen target cells. This is a mechanism that may explain how estradiol can exert diverse, highly selective effects in different target cells.

Concluding remarks and future perspectives

Estradiol is synthesized from testosterone by a cytochrome P450 aromatase present in the ovary and placenta and in

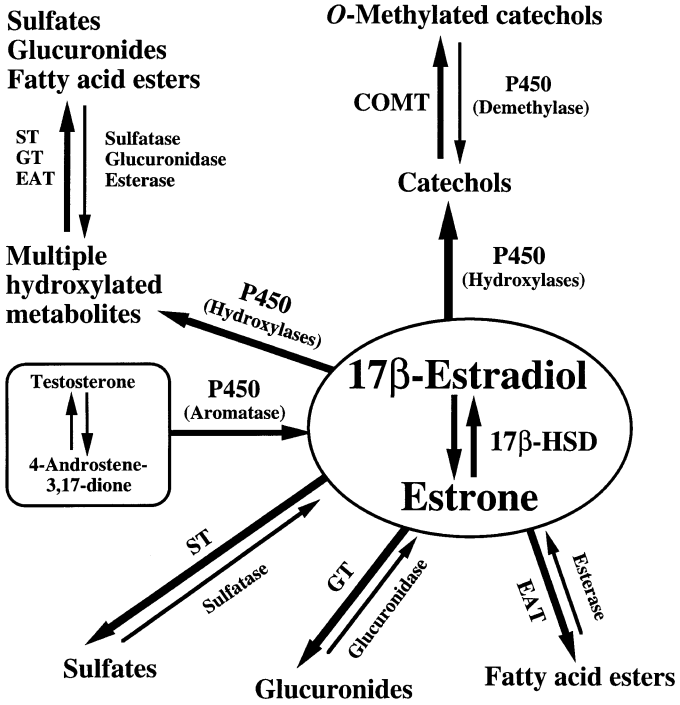

Fig. 5. Complexities of estrogen metabolism. *Abbreviations used:* ST (sulfotransferase), GT (glucuronosyltransferase), EAT (estrogen acyltransferase; for fatty acid ester formation), 17β-HSD (17β-hydroxysteroid dehydrogenase), COMT (catechol-*O*-methyltransferase) and P450 (cytochrome P450).

certain estrogen target tissues such as brain, prostate, uterus and mammary gland. Once formed, estradiol can be metabolized to multiple hydroxylated products by enzymes of the cytochrome P450 family, and 2- and 4-hydroxylated catechol estrogen metabolites can be *O*-methylated by catechol-*O*-methyltransferase. Estradiol and its hydroxylated metabolites can also be esterified to fatty acid esters or conjugated by glucuronidation or sulfonation which in turn may undergo de-esterification by esterase or deconjugation by β-glucuronidase or sulfatase to release active estrogens either in the liver or directly in target cells. This complexity of estrogen synthesis and metabolism (illustrated in Figure 5) provides many potential sites for the regulation of estrogen action.

Earlier studies demonstrated marked person-to-person differences in the metabolism of xenobiotics by cytochrome P450 enzymes (reviewed in 260,266,267,454), and these differences are caused by genetic (261–267) and environmental (109,110,260) factors such as drug administration, tobacco smoking, alcohol ingestion, pesticide exposure and dietary composition. Recent studies in humans revealed a large number of mutations in cytochrome P450 genes that impair xenobiotic metabolism (261,262,264–267,455,456), as well as the duplication of cytochrome P450 genes that results in super-fast metabolism of certain xenobiotics (263). Since steroid hormones are metabolized by many of the same cytochrome P450 enzymes that metabolize xenobiotics, changes in xenobiotic metabolism should also be reflected by an altered metabolism of steroid hormones. There is considerable variability in the levels of cytochrome P450 3A4 and 1A2 (cytochromes P450 prominent in catalyzing 2-hydroxylation of estradiol) in different human liver samples (up to 20-fold), and these differences provide an explanation for large interindividual variability in the hydroxylation of estradiol at C-2 and at other positions (112,118). Modulation of oxidative xenobiotic metabolism by cigarette smoking or by changing the ratio of dietary protein to carbohydrate in humans (110,260) also modulates the 2-hydroxylation of estradiol (277,457). Marked person-to-person variations have also been observed for the enzyme systems that catalyze conjugative estrogen metabolism (such as sulfonation; see refs 458,459) and for the *O*-methylation of catechol estrogens (see refs 422–426). Studies that identify genetic and environmental factors influencing estrogen metabolism at or near estrogen receptors in target cells may be of considerable importance since these factors could profoundly modify the biological effects of estrogens in complex manners depending on the pathways of metabolism that are affected and the biological activities of the metabolites that are formed. Such effects need not be associated with an altered profile of estrogen metabolites in the blood or urine.

Despite the widespread belief that increased exposure to estrogens contributes to the development of human mammary cancer, previous studies have only demonstrated a weak association of breast cancer with high estrogen intake or with a high circulating or urinary level of estrogen in women (251) although an elevated exposure to estrogens or high plasma level of estrogens is associated with a substantial increase in the risk for endometrial cancer (12–14,17,18). We believe that measurement of unmetabolized estrogens in plasma may be too crude a parameter to use successfully for relating exposure of target tissues to estrogens with the risk of cancer development. Several concepts should be stressed which may help our understanding of the underlying complexity of estrogen action: (i) The levels of unmetabolized estradiol and estrone in target tissues or in target cells within these tissues could be very different from estrogen blood levels. Previous studies reported that high concentrations of both albumin and estradiol are present in human mammary tumor cells (460,461). The presence of high levels of intracellular albumin (which has high binding affinity for estrogens) may significantly increase the intracellular levels of estrogens in mammary tumors. Moreover, an active transport of estrogen in addition to passive diffusion may also play a role in regulating the intracellular concentrations of estrogen (462). (ii) The hormonal activity of estrogens can be influenced by the activity of estrogen-metabolizing enzymes at or near estrogen receptors. In addition, metabolic formation of estrogen by aromatization of androgens and by deconjugation of estrogen conjugates in mammary tumor cells, as well as in the surrounding normal glandular and adipose cells may contribute to a high estrogen concentration in tumors. (iii) The carcinogenic activity of estrogens may be mediated or modulated by the formation of hormonally inactive or active metabolite(s) by specific estrogen-metabolizing enzymes in target cells. (iv) It is possible that unique biological effects of estrogen metabolites are mediated by specific receptors in target cells. In such cases, the formation of specific metabolites and the amounts of specific receptors in target cells may both be critical determinants for estrogen action.

It is of great interest that 2-methoxyestradiol was recently identified as an estradiol metabolite with strong activity as an inhibitor of cultured breast cancer cell proliferation *in vitro* and the growth of human mammary cancer cells *in vivo*. Further studies are needed to determine if physiologically formed 2-hydroxyestradiol and 2-methoxyestradiol have an inhibitory effect on estrogen-induced carcinogenesis.

In the present review, we suggest that certain estrogen metabolites may function as chemical mediators or as second-

ary hormones with unique functions. We point out that estrogen metabolism via multiple pathways can occur in target tissues at or near estrogen receptors. Estrogen metabolism in target tissues not only alters the intensity of estrogen action but may also alter its profile of physiological effects. Many factors modulate the levels of estrogen-metabolizing enzymes in liver and in target tissues, and the biological effects of an estrogen will depend on the profile of multiple metabolites formed and the biological activities of each of the metabolites. We believe that research on factors that influence the synthesis and metabolism of estradiol and its metabolites by diverse pathways at or near receptors in target cells is an underexplored area in need of more attention. Research in this area may lead to an enhanced understanding of estrogen action and to the discovery of pharmacologically active agents that are useful for the prevention and treatment of estrogen-induced cancers.

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