Chapter 6: Estrogen Metabolism by Conjugation

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The involvement of estrogens in carcinogenic processes within estrogen-responsive tissues has been recognized for a number of years. Classically, mitogenicity associated with estrogen receptor-mediated cellular events was believed to be the mechanism by which estrogens contributed to carcinogenesis. Recently, the possibility that estrogens might contribute directly to mutagenesis resulting from DNA damage has been investigated. That damage is apparently a result of the formation of catechol estrogens that can be further oxidized to semiquinones and quinones. Those molecules represent reactive oxygen species and electrophilic molecules that can form depurinating DNA adducts, thus having the potential to result in permanent nucleotide mutation. Conjugation of parent estrogens to sulfate and glucuronide moieties; of catechol estrogens to methyl, sulfate, and glucuronide conjugates; and of catechol estrogen quinones to glutathione conjugates all represent potential "detoxification" reactions that may protect the cell from estrogen-mediated mitogenicity and mutagenesis. In this chapter, the biochemistry and molecular genetics of those conjugative reaction pathways are discussed. When applicable, the involvement of specific enzymatic isoforms is presented. Finally, the activity of many of these conjugative biotransformation reactions is subject to large interindividual variation—often due to the presence of common nucleotide polymorphisms within the genes encoding those enzymes. Functionally significant genetic polymorphisms that might contribute to variable conjugation of estrogens and catechol estrogens are also discussed. [J Natl Cancer Inst Monogr 2000;27:113–24]

The involvement of estrogens in carcinogenic processes within the breast has been appreciated for a number of years *(1–3)*. The classical concept of estrogens as carcinogens recognizes the mitogenicity of estrogens via estrogen receptor (ER)-mediated cellular events *(1)*. More recently, as has been detailed throughout this monograph (Chapters 3–5), the role of catechol estrogens (CEs) as genotoxic chemical procarcinogens, independent of ER mediation, has been recognized *(2–4)*. Although estrogens and CEs differ with regard to the role of the ER in mediating their carcinogenicity, they have in common the potential for "detoxification" via enzyme-mediated conjugation to glucuronide, glutathione (GSH), methyl, and/or sulfate moieties *(2)*. In this chapter, we will discuss primary estrogen and CE conjugation reactions, with particular emphasis on the biochemistry and molecular genetics of the human enzymes that catalyze those reactions.

Estrogens exert biologic responses in steroid hormone-responsive cells largely via interaction with ERs, members of a superfamily of nuclear hormone receptors that act as ligand-activated transcription factors *(5)*. There are two known ER subtypes, ERα and ERβ , which share similar estrogen affinities but have dissimilar expression patterns and response to antiestrogens *(5–7)*. The two most potent endogenous estrogens, es-trone and 17β-estradiol, are both ligands for the ERs, although those receptors have higher affinity for 17β-estradiol than for estrone and it is 17β-estradiol that is believed to be the predominant endogenous activator of ER-mediated cellular processes *(5)*. The most abundant circulating estrogen, however, is the sulfate conjugate of estrone *(8,9)*. The process by which estrogens, synthesized and secreted predominantly by the ovaries, are transported to and exert their biologic effects in steroid hormone target tissues is not completely understood. As will be discussed in this chapter, estrogen conjugates, particularly estrone sulfates, are believed to play an important role in that process *(9–11)*.

Chemical carcinogenesis emerged as a scientific discipline approximately 50 years ago *(12,13)*. One of the principles of that discipline is that compounds often require metabolic "activation" to form genotoxic and carcinogenic metabolites *(12,13)*. That process involves the establishment of a balance between "activating" and "inactivating" metabolic pathways. The hypothesis that estrogens might contribute to the pathophysiology of breast cancer as direct genotoxins *(3,4)* has raised the possibility of just such a balance between estrogen activation and inactivation in those hypothetical genotoxic effects. Specifically, oxidative reactions, often catalyzed by isoforms of the cytochromes P450, can result in the formation of CEs from parent estrogens and, subsequently, semiquinones and quinones derived from CEs that are capable of forming either stable or depurinating DNA adducts *(14–16)*. Countering the effects of these pathways of metabolic activation are enzymatic reactions that inactivate the parent estrogens, the CEs, and quinones. Inactivation pathways involving conjugation reactions, such as methylation, sulfation, glucuronidation, or conjugation with GSH, will be detailed in this chapter. It is important to note that, although a number of animal models have been developed to facilitate the study of CE-mediated carcinogenesis, the focus of this chapter will be primarily on the conjugation of estrogens and CEs in humans. Although it has been hypothesized that conjugated CEs may exhibit biologic activity *(2)*, the focus of this chapter is on conjugation as a detoxification mechanism.

Finally, conjugation pathways of both estrogens and CEs display large variations among individuals—often as a result of common genetic polymorphisms. Therefore, the possibility arises that common, inherited variations in enzymatic pathways for estrogen bioactivation or in the inactivation of either the parent compound or downstream metabolites might represent individual risk factors for the occurrence of breast cancer. The

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See "Notes" following "References."

molecular epidemiology of estrogen carcinogenesis is detailed in Chapter 7. That chapter focuses on genetic polymorphisms that have been studied as risk factors for estrogen-mediated carcinogenesis. This chapter will present the current state of knowledge with regard to functionally significant genetic polymorphisms in human genes encoding estrogen-conjugating enzymes, many of them as yet untested as breast cancer risk factors.

ESTROGEN CONJUGATION

Biologic Role of Estrogen Conjugation

The endogenous formation of estrogen conjugates has long been recognized as a major route of estrogen metabolism (17). Both endogenous and synthetic exogenous estrogens are extensively biotransformed to estrogen conjugates in humans (Fig. 1) (2,18). The most abundant circulating estrogen conjugates are the sulfates, followed by the glucuronides. It is important to note that conjugated estrogens are not appreciable ligands for the ERs; thus, they do not promote ER-mediated activity (2). Intuitively, it was initially assumed that sulfate and glucuronide conjugation of estrogens represented a pathway resulting in less active, more polar, and more readily excreted estrogenic compounds. It is now appreciated, however, that estrogen sulfates actually exhibit a much longer half-life than do the parent estrogens (2,8,11). Estrone sulfate is the most abundant circulating estrogen, at concentrations approximately 10-fold higher than unconjugated estrone (8). That finding, as well as increasing knowledge about the transport and subsequent desulfation of estrogen sulfates, has led to a widely held belief that sulfated steroid hormones serve an important biologic role as steroid hormone precursors, particularly for steroid hormone-responsive tissues (2,9). An increasing body of scientific data supports the hypothesis that sulfation and desulfation of estrogens may well represent an endogenous system important in the regulation of biologically active steroid hormones in target tissues (10,11). Specifically, it is currently hypothesized that inactive estrone sulfate is transported to target tissues via the circulatory system, taken into target cells, most likely by organic anion transporters, enzymatically hydrolyzed to estrone by intracellular membrane-bound steroid sulfatase (arylsulfatase C), and hydroxylated to active 17β-estradiol via catalysis by 17β-hydroxysteroid dehydrogenases (2,11,18,19). 17β-Estradiol activates the ER via ligand binding and initiates a number of downstream ER-mediated events—most notably related to transcriptional activation of those genes that contain DNA sequences that bind and respond to activated ERs (5,18).

The transport of estrone sulfate into steroid hormone-responsive cells is not well understood; however, some studies (19,20) have shown that a human organic anion transporter (Oatp1) has high affinity for both sulfate and glucuronide estrogen conjugates. Furthermore, this transporter is typically responsible for intracellular import of organic ions rather than the efflux of these compounds out of the cell. The level of expression or activity of this transporter in human breast tissues has not yet been reported. Many target tissues including the breast exhibit estrogen sulfation activities in addition to the ability to desulfate estrogen sulfates (8,9). This "cycling" has been demonstrated in mammalian cells and, like phosphorylation and dephosphorylation of proteins during cell-signaling processes, sulfation and desulfation of steroid hormones possibly represent an intracellular regulatory mechanism for estrogenic activity

(Fig. 1) (8,11,21). Recognition of the importance of steroid sulfatase activity in the formation of intratumoral estrogens has resulted in the development of a number of steroid sulfatase inhibitors for the treatment of steroid hormone-responsive tumors (22,23).

Enzymes responsible for the glucuronidation and deglucuronidation of estrogens are also expressed in a variety of human tissues, including the breast (24,25). Estrogen glucuronides have received much less attention, however, than have the sulfate conjugates as steroid hormone precursors, most likely because they are less abundant and more readily cleared from the body (2). Breast tumors and breast cancer cell lines express high levels of β-glucuronidase, the enzyme that catalyzes the hydrolysis of estrogen glucuronides; however, appreciable estrogen glucuronide cycling in breast tissue has not been demonstrated (24). Although the concept of estrogen glucuronides as steroid precursors has been underinvestigated, it is generally accepted that glucuronidation of estrogens serves primarily a classical excretory role. Estrogen glucuronide conjugates are readily excreted in both urine and bile (26).

Biochemistry of Estrogen Conjugation

Sulfation. Sulfate conjugation of estrogens is catalyzed by several members of a superfamily of cytosolic sulfotransferase (SULT) enzymes (27,28). SULT enzymes catalyze the transfer of SO_3^- from 3′-phosphoadenosine-5′-phosphosulfate, the enzymatic cofactor, to, in the case of estrogens, phenolic acceptor groups (28). Cytosolic SULTs are active as homodimers. Sulfation of estrone and 17β-estradiol occurs at the 3-phenolic
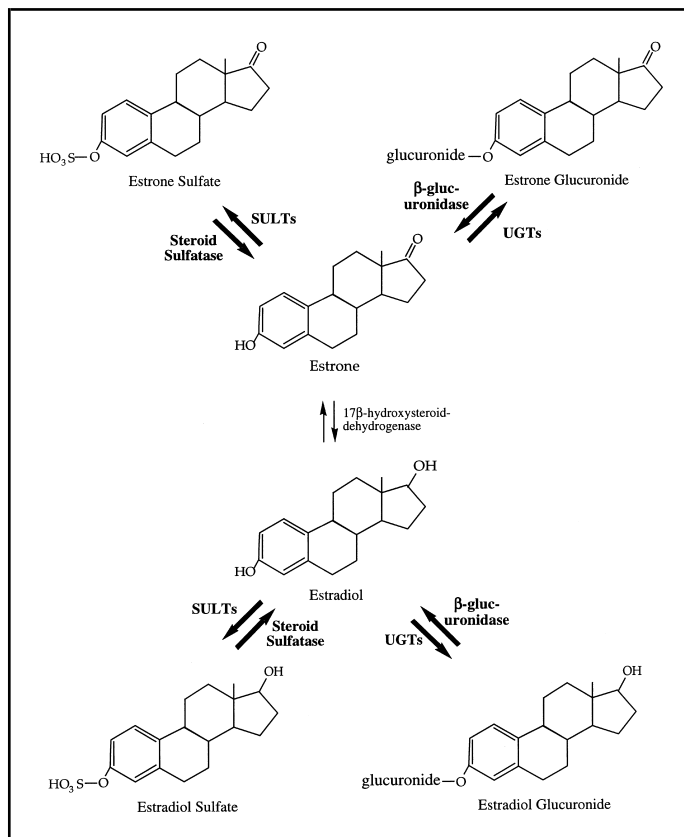

Fig. 1. Conjugative pathways for estrone and 17β-estradiol. Sulfation, desulfation, glucuronidation, and deglucuronidation reactions catalyzed by sulfotransferases (SULTs), steroid sulfatase, UDP-glucuronosyltransferase (UGTs), and β-glucuronidase, respectively, are shown.

group of the steroidal A ring (Fig. 1). Estrogen SULT activity has been demonstrated in a variety of human tissues, including liver, small intestine, kidney, placenta, uterus, adrenal gland, and breast *(29–33)*. The level of estrogen SULT activity in the human liver is high, and this activity is believed to contribute significantly to the high circulating levels of estrone-3-sulfate *(29,30)*. Although from a quantitative perspective, sulfation of estrogens in the liver is probably the most important overall estrogen-conjugating activity in the body, sulfation of estrogens in steroid target tissues, including the breast, has also been demonstrated and may well be important in affecting the biologic activity of estrogens within those tissues *(2,11)*.

Study of the association of estrogen SULT activity with breast cancer has been an active area of research. Expression of estrogen SULT activity within breast tumors has been reported to correlate with the ER status of the tumor as well as with the response of tumors to estrogens and adrenalectomy *(33,34)*. However, other studies have shown no such correlation or even an inverse correlation *(35)*. Such contradictory findings are indicative of the difficulty investigators have encountered in the study of estrogen sulfation in steroid target tissues. As will be discussed shortly, the reason for those difficulties has recently been appreciated in that we now know that multiple SULT enzymes contribute to estrogen SULT activity and, importantly, there is significant interindividual variation in the level of activity of the enzymes catalyzing the sulfation of estrogens *(27,30,36)*. Furthermore, SULTs are subject to profound substrate inhibition *(32)*. The concentration of substrate at which inhibition occurs differs among estrogen-sulfating isoforms such that slight differences in experimental conditions would have important implications in the interpretation of resulting data.

Glucuronidation. Estrogen glucuronidation is catalyzed by several members of a superfamily of microsomal UDP-glucuronosyltransferase (UGT) enzymes *(25,37)*. UGTs catalyze the conjugation of UDP-glucuronic acid, the UGT cosubstrate, to a variety of endogenous and exogenous aglycones, including steroid hormones *(38)*. Whereas estrogens are sulfated predominantly at the 3 position, glucuronidation can occur at either the 3 or 17β hydroxyl group of steroidal hormones, with the 17β position being the apparent predominant site of glucuronidation for 17β-estradiol (Fig. 1). Glucuronidation of estrogens renders those molecules less lipophilic and more readily excreted in both urine and bile. 17β-Glucuronides of estradiol are known to induce cholestasis, putatively via interaction with hepatocyte canalicular membrane efflux transporters such as MDR1 and MRP2/cMOAT *(39,40)*.

Steroid hormone glucuronidation has been observed in human liver, biliary epithelium, kidney, gut, prostate, ovary, and breast *(25,26,38)*. In a study comparing UGT activity in matched breast ductal carcinoma and peritumoral tissues, the authors *(24)* reported activity in tissues from only four of the 12 individuals studied. Furthermore, in those four sample pairs, the level of activity was fivefold lower in tumor tissue than in the peritumoral tissue. However, those studies were conducted with the use of 4-methylumbelliferone as substrate (as opposed to an estrogen), and it is not clear whether that activity correlates with estrogen glucuronidation in the breast. Glucuronidation is a major route of androgen metabolism, and the study of this pathway has received much attention in terms of its role in the pathophysiology of androgen-dependent diseases *(41)*. However, the role of estrogen glucuronides in breast cancer has received little attention compared with sulfate conjugation. This is most likely due to the perception that estrogen glucuronidation serves a predominantly excretory role, secondary to sulfate conjugation. It is clear that much further study of the glucuronidation of estrogens is required before we can fully understand the biochemistry of this pathway and its role in affecting estrogen activity.

Molecular and Cellular Aspects of Estrogen Conjugation

Biochemical studies of estrogen conjugation provided much knowledge about these important metabolic pathways. However, there were also many questions left unanswered by these studies, and we now have begun to be able to answer some of those questions using the tools and further knowledge gained with the advent of molecular biology. There are a surprising number of SULT and UGT isoforms capable of contributing to the conjugation of estrogens. Those isoforms are often expressed in a tissue-specific manner and are often under specific regulatory control. Furthermore, a number of those conjugative enzymes are encoded by genes known to harbor common genetic polymorphisms. These factors help explain many of the complexities of estrogen conjugation—and this knowledge allows us to probe estrogen conjugation reactions in a systematic fashion.

Sulfotransferases. The cloning of SULT genes and complementary DNAs (cDNAs) is a very active area of research *(27)*. Currently, there are at least 10 unique cytosolic SULT enzymes known to be expressed in human tissue *(27,42–45)*. On the basis of amino acid sequence identity, those 10 human SULTs fall within two families, SULT1 and SULT2. Subfamilies include SULTs 1A, 1B, 1C, 1E, 2A, and 2B. The 1A, 1C, and 2B families each have multiple members. Although amino acid identity allows the classification of these enzymes into families and subfamilies, members exhibit overlapping substrate affinities even across families. Estrone and 17β-estradiol are substrates for SULT1A1, SULT1E1, and SULT2A1, although the affinity of these enzymes for estrogens varies (Table 1) *(27)*. Overlapping substrate specificity of SULTs toward estrogens complicates the study of estrogen sulfation. For example, the high affinity of SULT1E1 for estrogen substrates suggests that this enzyme plays a major role in the endogenous sulfation of estrogens, and the activity of this enzyme in the liver likely contributes significantly to the quantitatively large pool of circulating estrogen sulfates *(29)*. It would be logical to hypothesize that this enzyme activity might be important in regulating estrogen activity in breast tumors. However, studies have suggested that, although SULT1E1 appears to be expressed in normal breast epithelial cells, it is not highly expressed in breast tumors or cell lines derived from breast tumors *(46)*. SULT1A1 and, to a lesser extent, SULT2A1 appear to be the SULT isoforms primarily responsible for estrogen sulfation in breast tumors *(46–48)*. These findings suggest that a specific SULT isoform may play a variable role in endogenous steroid hormone sulfation, depending on the tissue and the disease of interest.

Relatively little is known about the regulation of SULT genes. Although genes have been cloned for most of the human SULT cDNAs and enzymes identified to date, the DNA sequences contributing to the promotion or regulation of transcription of those genes have not been well defined. Of the human SULT genes cloned thus far, only SULT1E1 contains a canonical TATA box element, and experimentally determined sites of transcription initiation appear to correspond to the location of

Table 1. Specificity of sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT) isoforms with various estrogen and catechol estrogen substrates

Isoform	Substrate*					
	Estrone	Estradiol	2-OH-estrone	4-OH-estrone	2-OH-estradiol	4-OH-estradiol
SULTs						
1A1	X	X	ND	X	X	X
1E1	X	X	ND	ND	ND	ND
2A1	X	X	ND	—	—	X
UGTs						
1A1	—	X	X	X	X	X
1A3	X	ND	X	X	X	X
1A4	—	X	—	—	X	X
1A7	—	—	ND	—	X	ND
1A8	X	ND	X	X	X	X
1A9	X	X	X	X	X	X
1A10	X	X	ND	X	X	ND
2B4	—	—	ND	X	X	ND
2B7	—	—	X	X	X	X

*X = isoform has been shown to conjugate indicated substrate; — = isoform has been shown not to conjugate the indicated substrate; ND = interaction of the indicated isoform/substrate pair has not been determined. *See* text for details and references.

that element *(49)*. The level of estrogen SULT activity in human tissues has been reported to be under the influence of steroid hormones *(28)*. In concordance with that finding, the 5′-flanking region of the SULT1E1 gene contained half palindrome glucocorticoid and thyroid hormone response elements *(49)*. However, the functional significance of those elements has not yet been studied experimentally. Additional evidence of SULT regulation includes the identification of alternative sites of transcription initiation for the SULT1A1 gene *(50)*. The regulation or tissue selectivity of alternative transcriptional initiation of SULT1A1 has not been well studied.

Finally, conjugation of estrogens is known to vary significantly among individuals *(29,51)*. That observation raises the possibility that genetic variation in the genes contributing to estrogen conjugation (pharmacogenetics) may contribute to interindividual variation in estrogen metabolism. As will be discussed in Chapter 7, a number of genetic variants in genes contributing to estrogen metabolism have been reported to represent risk factors for the development of breast cancer. The study of the pharmacogenetics of SULT enzymes is currently an active endeavor. A common, functionally significant genetic polymorphism has been described for SULT1A1 *(52,53)*. That SULT1A1 polymorphism results in an Arg213His amino acid substitution. Correlation of the level of SULT activity in human blood platelet samples and SULT1A1 genotype suggests that individuals homozygous for the His allozyme exhibit a significantly diminished capacity to sulfate prototypic phenolic molecules *(52)*. The contribution of this polymorphism to interindividual variability in the conjugation of estrogens or as a risk modifier for breast cancer has not yet been reported.

Similarly, large interindividual variations in the level of SULT2A1 activity in human liver and the level of immunoreactive protein in intestinal tissues have also been reported *(30,54)*. Genetic polymorphisms resulting in Met57Thr and Glu186Val amino acid changes in SULT2A1 have been reported *(55)*. Functional studies of the recombinant SULT2A1 allozymes *(55)* have shown that those amino acid changes, particularly when coexpressed, result in a diminished level of recombinant enzyme activity. However, SULT2A1 genotype does not appear to correlate significantly with the level of apparent SULT2A1 activity in human tissues *(55)*. Finally, the presence

of large differences in the level of immunoreactive SULT1E1 protein in samples of human small intestines raises the possibility that genetic polymorphisms might also exist for that enzyme *(30)*. That possibility is currently the subject of active study, but no polymorphisms in the SULT1E1 gene have yet been reported.

Glucuronosyltransferases. As with the SULTs, a number of UGT isoforms are now known to contribute to the conjugation of estrogens (Table 1). The degree of contribution of individual UGTs to that activity is not yet well understood, and it is likely that specific isoforms will contribute differently, depending on the tissue and the disease of interest. There are currently at least 12 functional UGT isoforms known to be expressed in human tissues *(37)*. Like the SULTs, those 12 enzymes fall into two families within the human UGT superfamily of microsomal enzymes. Glucuronidation of estrone and 17β-estradiol appears to be catalyzed by several members of the UGT1 family. Thus far, recombinant human UGTs 1A1, 1A3, 1A4, 1A8, 1A9, and 1A10 have all been shown to catalyze the glucuronidation of estrone and/or 17β-estradiol (Table 1) *(25,26,56–60)*. It is interesting to note that, for some human recombinant UGT isoforms, there appears to be selective affinity for estrone or 17β-estradiol as substrate. For example, UGTs 1A1 and 1A4 exhibit activity toward 17β-estradiol but not toward estrone, whereas UGTs 1A9 and 1A10 have been reported to catalyze the glucuronidation of both of these estrogens *(25,26,56,58,60)*. Activity for UGTs 1A8 and 1A3 toward estrone has been reported, but the activity of those isoforms toward 17β-estradiol has apparently not been evaluated *(57,59)*.

There is much known about the expression of human UGT1A isoforms in various tissues. It should be noted, however, that the tissue distribution profile of some UGT isoforms has not been as extensively characterized as others. UGT1A1 is expressed in human liver, colon, and biliary epithelium (gallbladder) but not in stomach *(60)*. UGT1A3 has been reported to be expressed in human colon, biliary epithelium, and liver, but the level of expression in liver varied significantly between individuals and was fivefold to 10-fold less than the level of UGTs 1A1 and 1A4 *(57,60)*. UGT1A4 is expressed in human liver, colon, and biliary epithelium but not in stomach *(60)*. UGT1A8 appears to be expressed specifically in human intestinal tissues *(59,60)*. UGT1A9 is expressed in human prostate, testis, breast, ovary,

skin, skeletal muscle, stomach, small intestine, colon, liver, and kidney but not in biliary epithelium or stomach *(25,60)*. UGT1A10 is expressed in colon, biliary epithelium, and stomach but not in liver *(60)*. It is important to note that, although only the UGT1A9 isoform has been reported to be expressed in human breast to date, that is likely a reflection of the lack of evaluation of the level of expression of various UGT isoforms in that tissue.

The regulation of the UGT1 family is currently not well characterized but is an active area of study. The most notable feature of this gene family is that all of the UGT1A isoforms disseminate from a single "nested" gene structure *(37)*. There are six coding exons in the human UGT1A genes, and each isoform is encoded by the same exons 2 through 5. The only differentiation between isoforms is that each exon 1, encoding the N-terminal half of the protein, is unique, and isoform specificity results from alternative transcription initiation and usage of unique exons 1 *(37)*. Therefore, each isoform is under the control of individual promoter sequences, and isoform-specific regulation has been observed. For example, as noted in the previous paragraph, UGT1A isoforms are differentially expressed in human tissues.

As previously noted, the capacity for estrogen conjugation and, specifically, glucuronidation is known to vary widely in the human population *(51)*. That observation raises the possibility that genetic variation may exist in the UGT isoforms that contribute significantly to estrogen glucuronidation. A functionally significant common polymorphism in the promoter sequence of the UGT1A1 gene has been described and well characterized *(61–63)*. That polymorphism is a variable length $(TA)_n$ TAA repeat in the functional TATA box upstream of exon 1 of the UGT1A1 gene. The wild-type allele is defined as n = 6. Allelic variants identified to date include n = 5, 7, and 8 *(63)*. *In vitro* studies utilizing reporter constructs driven by allelic variants of the UGT1A1 promoter *(63)* have shown that promoter activity appears to decrease with increasing n. Furthermore, clinical association of the most common variant (n = 7) with a relatively poor ability to glucuronidate bilirubin (Gilbert's syndrome), as well as the chemotherapeutic agent SN-38, has been observed *(61,64)*. Studies determining the association of UGT1A1 alleles with estrogen metabolism and risk modification of breast cancer have not yet been reported, but they will be of great interest.

CE CONJUGATION

### Biologic Role of CE Conjugation

The putative role of CEs in the mediation of breast carcinogenesis has been described in Chapters 3–5 of this monograph. The biotransformation of estrone and estradiol to CEs involves hydroxylation at the 2 or 4 position of the steroidal A ring of these parent estrogens *(3,14)*. Those reactions are catalyzed by multiple cytochrome P450 isoforms. Both the 2- and the 4-hydroxy CEs can be further oxidized to CE quinones (CE-Qs) or semiquinones (Fig. 2) *(16)*. The 2-hydroxy CE-Qs have been shown to form stable DNA adducts, whereas the 4-hydroxy CE-Qs have been shown to form depurinating adducts *(16,65,66)*. There is good evidence suggesting that those depurinating adducts can lead to apurinic DNA sites and permanent mutations that, when inflicted upon critical DNA sequences, can lead to tumorigenesis *(16,66)*. CEs can also enter into redox cycling

and, thereby, become a source of reactive oxygen species *(3)*. Hence, unless CEs are inactivated, they may contribute to carcinogenesis by causing DNA damage mediated by reactive oxygen species and by direct interaction of CE-Qs with DNA to form depurinating adducts *(65,66)*. Fortunately, our cells are fortified with an armament of conjugative pathways that result in the biotransformation of toxic estrogen metabolites to relatively nontoxic moieties *(2)*. Generally, the reactive CEs are detoxified by biotransformation to predominantly methyl conjugates, to a lesser extent glucuronides, and possibly sulfate conjugates (Fig. 2) *(2)*. A further conjugative safeguard lies in the detoxification of CE-Qs via conjugation to glutathione *(16,67)*. Therefore, the actual risk of CEs in causing DNA damage may well depend on the ability of individual cells to conjugate CEs and CE-Qs relative to the rate of formation of these toxic estrogen metabolites. In the rest of this chapter, we will focus on CE conjugative pathways and those conjugative enzymes responsible for the detoxification of CEs and CE-Qs.

The most well-studied CE conjugation reaction is that of methylation. CE methylation is catalyzed by catechol-*O*-methyltransferase (COMT), an enzyme that exists in both "soluble" (S-COMT) and membrane-bound (M-COMT) forms, as discussed in detail below *(16,68)*. Studies in the hamster kidney *(69)* provided the first example linking an estrogen-induced cancer with the induction of COMT. The localization of COMT in the epithelial cells of the proximal convoluted tubules of the hamster kidney is similar to its localization in the rat kidney reported earlier *(70)*. Hamsters treated with primary estrogens, such as estrone and estradiol, develop tumors in the renal cortex. There is evidence that the carcinogenicity of estrogens for hamster kidney results from a combination of factors: 1) an increase in the catechol load, 2) the presence of high levels of 2- and 4-hydroxylated CEs subject to oxidative metabolism in the renal cortex, and 3) a relative insufficiency of COMT *(71)*. In control hamsters, COMT was localized in the cytoplasm of proximal convoluted tubules, predominantly in the juxtamedullary region where estrogen-induced tumors arise. After 2 or 4 weeks of treatment with estrogen, COMT was seen in epithelial cells of the proximal convoluted tubules throughout the cortex. Moreover, many cells showed intense nuclear COMT immunoreactivity (Fig. 3) *(69)*. The estrogen-induced cancers were COMT negative but were surrounded by tubules with epithelial cells with intense cytoplasmic and nuclear immunostaining. Immunoblot analysis indicated that the nuclear COMT, shown in Fig. 3, was S-COMT. This translocation to the nucleus was shown by sequencing of hamster kidney COMT messenger RNA to occur in the absence of a nuclear localization signal. This pattern of induction of COMT in hamster kidney in response to estrogen treatment, in particular in the nucleus, has been interpreted as a possible response to "a threat" to the genome by products of oxidative metabolism of CEs.

It is of interest that nuclear localization of COMT is not unique to hamster kidney but also can be seen in some normal, as well as neoplastic, mammary epithelial cells *(72)*. Human breast tissues have the capacity to synthesize both 2- and 4-hydroxyestrogens *(71,73)*. A cytochrome P450 that catalyzes the 2- and 4-hydroxylation of estrogen has been identified by immunocytochemistry in human ductal epithelial cells *(74)*. COMT has also been identified in those cells *(75)*. High levels of oxidatively damaged DNA have been found in breast tissue from women in the United States *(76,77)*. It is reasonable to propose

Fig. 2. Conjugation of estrogens, catechol estrogens, and estrogen quinones. Reaction pathways and the enzymes involved are shown. For simplicity, the formation of estrogen semiquinones is not depicted. (*See* Chapter 5 for details.) COMT = catechol-*O*-methyltransferase; SULTs = sulfotransferases; UGTs = UDP-glucuronosyltransferases; CYPs = cytochromes P450; GSTs = glutathione *S*-transferases.
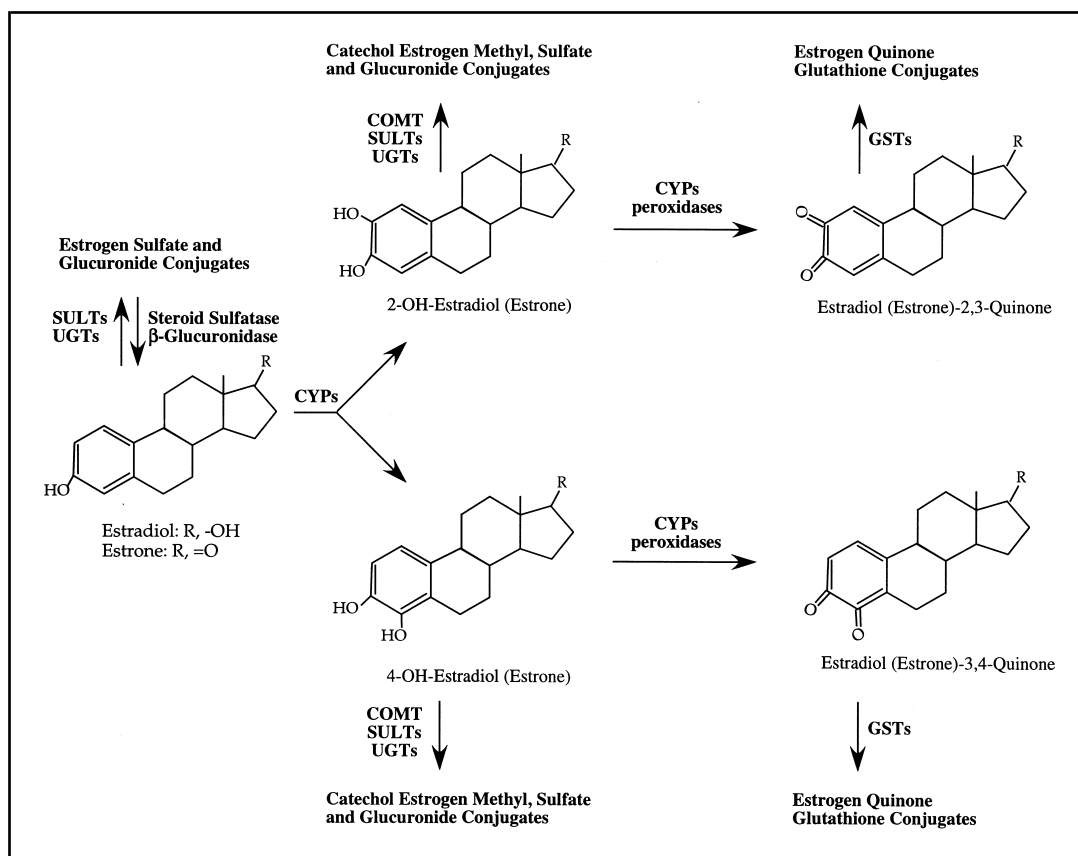
that, in human breast tissue, like the kidneys of hamsters treated with estrogen, oxidative metabolism of CEs might contribute to this oxidative damage.

Biochemistry of CE Conjugation

Methylation. Quantitatively, the most active CE conjugative pathway is methylation. CE methylation is catalyzed by COMT, a member of a superfamily of methyltransferase enzymes *(68)*. COMT, a classical phase II enzyme, catalyzes the transfer of methyl groups from *S*-adenosyl methionine, the enzyme cofactor, to hydroxyl groups of a number of catechol substrates, including the CEs. Under normal circumstances, CEs are, for the most part, promptly *O*-methylated by COMT to form 2- and 4-*O*-methylethers, which are then excreted *(78)*. While virtually all catechols are substrates for COMT, the highest affinities for the enzyme are exhibited by the CEs *(78)*. The existence of this metabolic pathway helps to explain the extremely short half-life of CEs and the predominance of *O*-methylethers of CEs, in particular of 2-methylethers, as the major metabolites of estrone and estradiol in urine *(79)*. However, under circumstances during which the capacity for *O*-methylation is reduced or inhibited by an excess catechol load, the half-life of CEs may be extended. This phenomenon could have special importance for specific cellular sites, such as breast epithelial cells, where CEs are formed. COMT might play an important role in protecting the genome from damage that could be caused by the metabolism of estrogens through activation of the CE-Q pathway. A number of investigators are now studying the involvement of this enzyme as well as the interindividual variability of COMT enzyme activity in detoxification of CEs specifically in the context of breast carcinogenesis.

The hypothesis that COMT provides a protective mechanism against cytotoxicity and genotoxicity by preventing the oxidation of catechols is in its infancy. At present, we know enough to consider *O*-methylation an important mechanism for preventing cytotoxic and genotoxic damage caused by products of the oxidative metabolism of catechols. This knowledge may generate avenues for therapeutic intervention where a deficit in the capacity for *O*-methylation appears to be a risk factor in carcinogenesis *(80,81)*.

Sulfation and glucuronidation. While methylation of CEs has been well studied, very little is known about the role of sulfation and glucuronidation in the detoxification of CEs. The excretion of both sulfate and glucuronide conjugates of CEs has been observed in rats *(82)*, and it is clear from a number of *in vitro* studies that UGTs and SULTs are able to catalyze the conjugation of CEs. We also know that those enzymes are expressed in the liver and estrogen-responsive tissues, such as the breast epithelium *(25,27)*. Therefore, it is plausible to suggest that these reactions may play a biologic role in the detoxification of CEs. A number of studies [reviewed in *(2)*] have reported that the major urinary metabolites of CEs are the methyl conjugates. From a quantitative perspective, therefore, the formation of sulfate and glucuronide CEs does not appear to represent major pathways in the overall metabolism and excretion of CEs. However, because the reactivity and toxicity of the CEs are intracellular phenomena, it has been suggested that local metabolism of CEs within target cells will be just as important as the overall detoxification of CEs in the liver *(2)*. For that reason, a number of investigators are now studying the role of sulfate and glucuronide conjugation of CEs in the intracellular detoxification of these carcinogens. COMT, UGTs, and SULTs often share affin-
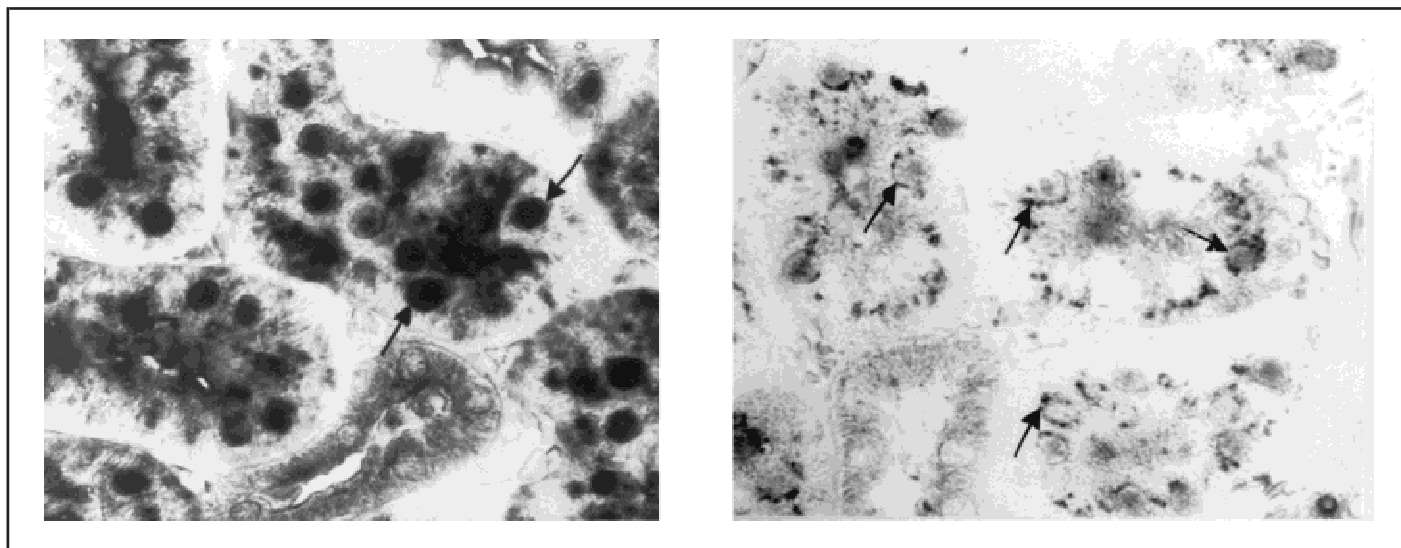
Fig. 3. Immunoreactive catechol-*O*-methyltransferase (COMT) (**left**) and CuZn-superoxide dismutase (CuZnSOD) (**right**) in proximal convoluted tubules from the same region in adjacent tissue sections from the kidney of a hamster treated for 2 weeks with estradiol (original magnification ×500). Intense immunostaining for COMT is seen with many nuclei in contrast to the perinuclear immunostaining for CuZnSOD. **Arrows** point to some of the cells with distinct immunostaining for COMT and perinuclear staining for CuZnSOD. This figure is used by permission of Oxford University Press from *Carcinogenesis (69)*.

ity for the same substrates. An indirect role that has been suggested for the relevance of SULTs and UGTs in the detoxification of CEs is that those enzymes might represent pathways for the conjugation of other catechol substrates that may compete for, and thus inhibit, the capacity of COMT to detoxify CEs *(2)*. Those hypotheses have yet to be rigorously tested at the experimental level.

GSH conjugation. The reactivity of CE-Qs relates to their ability to undergo redox cycling, creating oxidative stress, and/or to react directly with cellular nucleophiles (such as DNA) *(3,16)*. Conjugation of quinones to GSH, a major cellular sulfhydryl tripeptide, is generally considered a detoxification mechanism *(16,83)*. GSH conjugation of CE-Qs has been shown to occur both *in vivo* and *in vitro (84)*. GSH-conjugated CE-Qs are then rapidly converted to mercapturic acid metabolites that are readily excreted from the cell. It is primarily this excretory role of GSH conjugation that is believed to contribute to the detoxification of CE-Qs. However, the actual degree of detoxification of CE-Qs that is imparted by GSH conjugation is unclear because GSH-conjugated quinones are capable of undergoing the same redox cycling reactions as are the parent quinones and semiquinones *(83,84)*. Those reactions result in the formation of reactive oxygen species that can themselves cause DNA damage. Therefore, the "net" protective effect of conjugation of CE-Qs by GSH depends on the relative balance between GSH-mediated CE excretion and the GSH-mediated formation of reactive oxygen species. Most studies appear to confirm that conjugation of CE-Qs with GSH results in a net decrease in DNA damage *(67,85)*.

Molecular and Cellular Aspects of CE Conjugation

Catechol-*O*-methyltransferase. A single gene encoding COMT is expressed at the protein level in two forms as a consequence of the existence of alternative transcription initiation sites *(86)*. The two transcriptional products result in the translation of an S-COMT and an M-COMT enzyme with M_r values of 23 000 and 26 000 daltons, respectively. M-COMT includes an additional 50 amino acid residues at the N-terminus of the protein that are not present in S-COMT *(86,87)*. Of the two forms, the cytosolic S-COMT has a lower affinity but a higher capacity for catecholamines than M-COMT *(68)*. The relative expression of the two COMT enzymes varies with different tissues, but S-COMT appears to be the dominant form in most cell populations *(87–89)*. COMT is widely distributed, with high levels of activity being reported in the liver and kidney epithelium, as well as in the ependymal and glial cells. In breast tissue, immunoreactive COMT has been observed in both normal and neoplastic epithelial cells *(75)*. In neoplastic cells of rodent and human breast, COMT enzyme activity, expressed as units per milligram of protein, has been reported to be elevated *(75,90)*. However, this apparent increase may be due to an increase in cell numbers in neoplastic breast parenchyme.

Extensive cytochemical studies of the localization of COMT both at the cellular and subcellular levels *(91)* support the hypothesis that COMT plays a critical role in the local regulation of catechols at specific target sites. Regulation of COMT expression appears to be tissue selective and site specific. In liver and possibly in red blood cells, COMT functions in the *O*-methylation of circulating endogenous and xenobiotic catechols *(92)*. In addition, in liver, quantitatively the most important site for the metabolism of estrogens via 2-hydroxylation, COMT serves to inactivate 2-OH CEs close to the site where they are formed. In many other tissues in which COMT is expressed, it appears to have a critical role in restricting the passage of catechols between tissue compartments *(93)*. An example is the dense concentration of COMT in the epithelial cells of the choroid plexus that separate the vascular system from the spinal fluid. Another example is the presence of COMT in ependymal cells lining brain ventricles separating the spinal fluid from the brain parenchyma. The presence of COMT in astrocytes, oligodendrocytes, and microglia may well restrict the movement of catechols to "fields" in the central nervous system. In certain tissues, the expression of COMT has been shown to be under hormonal control.

Studies of the expression of COMT in the rat uterus provide

an example of a precise spatial and temporal expression of COMT and of its hormonal regulation in relation to a critical physiologic event, implantation *(94)*. Immunoreactive COMT becomes evident in the luminal epithelium of the uterus at the site of decidualization just before implantation on day 3 of pregnancy. The role of progesterone in the induction of COMT was demonstrated by the effective blockade of enzyme expression by RU-486 *(95)*. Since there is evidence that CEs generated in the uterus may have an important role in the process of implantation, the induction of COMT by progesterone could serve to delimit the action of CEs to the implantation site *(96)*.

Finally, levels of COMT activity in humans were shown more than 20 years ago to be controlled, in part, by a common genetic polymorphism *(97)*. The phenotypic trait of low COMT activity was found in approximately 25% of a Caucasian population. Molecular pharmacogenetic studies *(98)* have identified a single nucleotide polymorphism in the COMT gene that results in a Val108Met (amino acid 108 in S-COMT) amino acid substitution. This amino acid change is of great functional significance, since the methionine substitution results in a protein with low enzyme activity, and correlation of low COMT activity with COMT genotype has been reported in human tissues. It is notable that this COMT genetic variant represents a truly "balanced" polymorphism, in that the frequency of occurrence of each allele is approximately 50%. The description of the molecular genetic basis for low COMT activity made possible genetic epidemiologic studies and, as pointed out in Chapter 7, COMT has been a focus for studies of the genetic epidemiology of breast cancer. Unfortunately, the results of those studies *(80,81,99)* are conflicting. Therefore, these two complementary issues serve to illustrate—in both a broad and a highly focused fashion—the promise and limitations of this overall research strategy. This approach almost certainly will be applied with increasing frequency to help elucidate the possible contribution of direct estrogen genotoxicity to the pathophysiology of breast cancer and other neoplasia.

Sulfotransferases and UDP-glucuronosyltransferases. The role of sulfation and glucuronidation as detoxification pathways for CEs is underinvestigated. However, catalysis of CE conjugation by human recombinant SULT and UGT enzymes has been reported. Those results will be presented here, but it should be cautioned that the relevance these studies have to *in vivo* CE conjugation is not yet clear. SULT1A1, in addition to catalyzing the sulfation of estrone and estradiol, also catalyzes the sulfation of 4-hydroxyestrone, as well as of 2- and 4-hydroxyestradiol (Table 1) *(100)*. In that same study, SULT2A1 was reported not to catalyze the sulfation of 2-hydroxyestradiol or 4-hydroxyestrone but to have marginal activity toward 4-hydroxyestradiol. There have apparently been no other reports of specific SULTs catalyzing the sulfation of CEs.

It is tempting to speculate that SULT1A3, a catechol-preferring SULT, or SULT1E1, an estrogen SULT, might participate in the sulfation of CEs. Reverse transcription–polymerase chain reaction studies have suggested that SULT1A3 was highly expressed in human breast tumors and cell lines relative to the expression of SULT1A1 (Raftogianis R: unpublished data). However, it is not yet known whether SULT1A3 contributes to the sulfation of CEs. It has also been suggested that SULT1A3 might indirectly contribute to the regulation of CE conjugation by sulfating other catechols that would otherwise compete for COMT *(2)*, thus inhibiting CE methyl-ation. Hypotheses involving the role of SULT1A3 in CE conjugation have yet to be rigorously tested experimentally. SULT1E1 is expressed in normal breast epithelium, but it is not known whether that enzyme catalyzes the sulfation of CEs *(46)*.

A common polymorphism has been described for SULT1A1 *(52,53)*, and a number of laboratories are currently testing the hypothesis that this polymorphism may represent a risk factor for breast cancer. SULT1A1 polymorphisms are hypothesized to modify susceptibility to estrogen-mediated carcinogenesis via both sulfation of parent estrogens and variable detoxification of CEs (Figs. 1 and 2). Finally, biochemical pharmacogenetic studies *(101)* have shown that a common genetic polymorphism results in interindividual variation in the activity of SULT1A3. However, there have been no reports on the molecular basis for this polymorphism. Should SULT1A3 be involved in the detoxification of CEs, it is possible that polymorphisms in this gene might represent risk factors for susceptibility to CE-mediated breast cancer.

A large number of human recombinant UGTs, from both the UGT1 and UGT2 families, catalyze the glucuronidation of CEs (Table 1). Although there is much substrate overlap among these isoforms, there does appear to be some selectivity of isoforms toward specific CEs. UGT1A1 and UGT1A3 both catalyzed the conjugation of 2- and 4-hydroxy CEs, with particularly high activity toward the 2-hydroxy CEs *(102)*. UGT1A4 exhibited low levels of activity toward 2- and 4-hydroxyestradiol and no activity with estrone CEs *(58)*. UGT1A7 has been shown to catalyze the glucuronidation of 2-hydroxyestradiol *(60)*. UGT1A8 and UGT1A9 have also been reported to conjugate all four CEs, but with particularly high activity toward the 4-hydroxy CEs *(25,59)*. In a separate publication *(60)*, however, UGT1A8 was reported not to catalyze the conjugation of 2-hydroxyestradiol or 4-hydroxyestrone. UGT1A10 catalyzed the conjugation of 2-hydroxyestradiol and 4-hydroxyestrone *(60)*. In the UGT2 family, the recombinant enzymes for both UGT2B4 and UGT2B7 catalyzed the glucuronidation of CEs *(102–104)*. UGT2B4 (previously referred to as 2B11) catalyzed the conjugation of 4-hydroxyestrone and 2-hydroxyestradiol *(104)*. UGT2B7 exhibited activity toward the 2- and 4-hydroxy CEs, with particularly high activity toward the 4-hydroxy CEs *(102,103)*.

In addition to the functional variable repeat polymorphism in the TATA box already discussed for UGT1A1, common polymorphisms exist in both UGT2B4 and UGT2B7. The UGT2B4 polymorphism is defined by an Asp458Glu amino acid substitution that results in a protein with diminished UGT activity *(105)*. The UGT2B7 polymorphism causes a His268Tyr amino acid change that apparently does not alter the function of UGT2B7 *(102)*. Whether the UGT1A1 or 2B4 polymorphisms result in clinically significant variation in the *in vivo* conjugation of CEs is not yet known.

Glutathione *S*-transferases. Members of a superfamily of cytosolic GSTs catalyze the conjugation of GSH, the reactive cosubstrate, to a variety of electrophiles *(106)*. Although GSH conjugation can occur independent of GST-mediated catalysis, GSTs likely play a role in the catalysis of GSH conjugation of CE-Qs. GSTs are a major class of detoxification enzymes. There are estimated to be at least 20 human GST isoforms *(106)*. Their activity has been associated with the inactivation of a large number of xenobiotics, including many drugs. The ability of many tumors to exhibit increased levels of intracellular GST

expression has been implicated as a mechanism of chemotherapeutic drug resistance *(107)*. GST enzymes are encoded by a superfamily of GST genes *(106)*. The nomenclature adopted for this superfamily is quite different from that for the cytochromes P450, SULTs, or UGTs. The five families of GSTs have been designated GST alpha (α), mu (μ), pi (π), sigma (σ), and theta (θ). Humans possess a single functional GSTπ, but each of the other families contains multiple family members. GST enzymes are active as either homodimers or heterodimers. The frequent occurrence of functional GST heterodimers has made the study of substrate specificity for particular GST isoforms difficult. Perhaps it is for this reason that there is a lack of reports regarding the specific GST isoforms that contribute to the formation of CE-Q–GSH conjugates.

There is much known about the molecular genetics of human GSTs *(106)*. Many GSTα isoforms are expressed in human liver and skin, while some are ubiquitously expressed. Some members of the GSTμ family are expressed in human liver, while others are expressed in muscle, testis, brain, and heart. GSTπ is ubiquitously expressed, and GSTθ has been reported in human liver and red blood cells. There have been a number of reports indicating the high inducibility of GSTs by a variety of agents. Polycyclic aromatic hydrocarbons, phenolic antioxidants, reactive oxygen species, barbiturates, and synthetic glucocorticoids have been shown to induce GSTs. Induction of GSTπ has been of particular interest because of its putative role in drug resistance *(107)*. The mechanisms by which GSTs are inducible are apparently diverse *(106)*. The regulation of GST expression appears to be quite complex. A number of genetic response elements have been characterized in GST genes, including xenobiotic, antioxidant, and glucocorticoid-responsive elements. Furthermore, GST subunit expression is quite tissue specific, and regulatory elements contributing to tissue specificity are beginning to be defined. An NF-κB-like repressor element has recently been described in the human GSTπ gene. Expression of GSTs also appears to undergo sex- and age-specific regulations.

A number of genetic polymorphisms have been described for human GSTs, including variations in the GSTμ, GSTπ, and GSTθ genes *(106,108)*. The most notorious GST polymorphism is the null gene for GSTμ *(106)*. This polymorphism is defined by a deletion of the GSTM1 (μ) gene. The frequency of homozygosity of this deletion varies with ethnicity, from approximately 22% in Nigerians to 58% in Chinese populations. Epidemiologic studies have suggested that individuals who are null for the GSTM1 gene may be at increased risk for a variety of neoplastic diseases. The epidemiology of this polymorphism in breast cancer is discussed in Chapter 7 of this monograph. A common single nucleotide polymorphism in the human GSTπ gene resulting in an Ile105Val amino acid substitution has been identified, and the Val104 variant is associated with low GSTπ activity *(108)*. In addition, the Val104 allele has been associated with increased risk for prostate cancer. Epidemiologic studies of the role of this polymorphism in breast cancer are discussed in Chapter 7. An additional null allele for a GSTθ gene, GSTT1, has also been reported *(106)*. The frequency of the homozygous GSTT1 null genotype has been reported to vary from 16% in British Caucasians to 38% in Nigerians. The biologic consequences of the GSTT1 null genotype are not yet clear, but studies of this polymorphism and breast cancer susceptibility are also discussed in Chapter 7.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Conjugation is clearly a major biotransformation pathway for estrogens in humans. Recognition of the contribution of estrogen conjugation and deconjugation in breast cancer has been a relatively recent event. Increasing evidence suggests that the role of estrogen conjugation, particularly sulfation, goes beyond that of an excretory function and is perhaps even a major regulator of biologically active estrogens. Much less is known about conjugation of CEs, but the role that these conjugative pathways play in the biotransformation of CEs is an emerging story. Methylation of CEs appears to be an important detoxification mechanism, and some evidence suggests that variation in the capacity of cells to methylate CEs may represent a risk factor for susceptibility to breast cancer.

Clearly, more investigative effort will be required to fully understand which, if any, of these conjugative pathways modify cancer susceptibility or progression. As described in the next chapter, the study of low-penetrance, risk-modifying genes is very active, and we are beginning to see the inclusion of genes, such as COMT, that contribute to estrogen and CE conjugation among those being studied. As more genetic polymorphisms in estrogen- and CE-conjugating enzymes are identified, even larger epidemiologic studies will be necessary to delineate which of these variations or—more likely—which set of these genetic variants, represent cancer risk factors. The identification of novel genes encoding conjugating enzymes and "functionally significant" polymorphisms within those genes is occurring at a rapid pace. New molecular information arising from this era of "functional genomics" will require careful biochemical and large-scale epidemiologic studies before we can understand the biologic interplay and ultimate cellular consequence of the apparent myriad biotransformations that estrogens undergo and how these reactions contribute to carcinogenesis.

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