Sequential Action of Phase I and II Enzymes Cytochrome P450 1B1 and Glutathione S-Transferase P1 in Mammary Estrogen Metabolism

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ABSTRACT

The Phase I enzyme cytochrome P450 1B1 (CYP1B1) has been postulated to play a key role in estrogen-induced mammary carcinogenesis by catalyzing the oxidative metabolism of 17β-estradiol (E2) to catechol estrogens (2-OHE1 and 4-OHE2) and highly reactive estrogen quinones (E2-2,3-Q and E2-3,4-Q). The potential of the quinones to induce mutagenic DNA lesions is expected to be decreased by their conjugation with glutathione (GSH) either nonenzymatically or catalyzed by glutathione S-transferase P1 (GSTP1), a Phase II enzyme. Because the interaction of the Phase I and Phase II enzymes is not well defined in this setting, we prepared recombinant purified CYP1B1 and GSTP1 to examine their individual and combined roles in the oxidative pathway and used gas and liquid chromatography/mass spectrometry to measure the parent hormone E2, the catechol estrogens, and the GSH conjugates. 2-OHE1 and 4-OHE2 did not form conjugates with GSH alone or in the presence of GSTP1. However, incubation of GSH and CYP1B1 with 2-OHE2 resulted in nearly linear conjugation through C-4 and C-1 (i.e., 2-OHE4, 4-SG and 2-OHE1, 1-SG), whereas the reaction of 4-OHE2 yielded only 4-OHE2, 2-SG. When CYP1B1 and GSTP1 were added together, the rate of conjugation was accelerated with a hyperbolic pattern of product formation in the order 4-OHE2, 2-SG > 2-OHE2, 4-SG > 2-OHE1, 1-SG. Incubation of E2 with CYP1B1 and GSTP1 resulted in the formation of 4-OHE2, 2-OHE1, 4-OHE2, 2-SG, 4-OHE2, 4-SG, and 2-OHE1, 1-SG. The production of GSH-estrogen conjugates was dependent on the concentrations of E2 and GSTP1 but overall yielded only one-tenth of the catechol estrogen production. The concentration gap between catechol estrogens and GSH-estrogen conjugates may result from nonenzymatic reaction of the labile quinones with other nucleophiles besides GSH or may reflect the lower efficiency of GSTP1 compared with CYP1B1. In summary, both reactions are coordinated qualitatively in terms of product formation and substrate utilization, but the quantitative gap would leave room for the accumulation of estrogen quinones and their potential for DNA damage as part of estrogen-induced mammary carcinogenesis.

INTRODUCTION

Estrogens have been implicated in the development of breast cancer by simultaneously stimulating cell proliferation and gene expression via the estrogen receptor and by causing DNA damage via their genotoxic catechol estrogen metabolites (1, 2). The latter are produced in a series of linked oxidation reactions that have been proposed by several investigators to form the oxidative estrogen metabolism pathway (3, 4). The pathway starts with the two major estrogens, E2 and E1, which are oxidized to the 2-OH and 4-OH catechol estrogens by the Phase I enzymes CYP1A1 and CYP1B1 (5–7). These enzymes are postulated to further oxidize the catechol estrogens to unstable semiquinones and quinones. The estrogen quinones then form Michael addition products with deoxyribonucleosides (8–10). Furthermore, catechol estrogens and their estrogen quinones/semiquinones undergo redox cycling, which results in the production of reactive oxygen species capable of causing oxidative DNA damage (11–16). Thus, P450-mediated estrogen metabolism is expected to lead to the formation of both oxidative and estrogen DNA adducts, all of which have been shown to possess mutagenic potential (17–20).

It is postulated that the genotoxicity of the oxidative estrogen metabolism pathway is reduced by alternate reactions of the metabolites with Phase II enzymes. Specifically, catechol-O-methyltransferase catalyzes the methylation of catechol estrogens to methoxy estrogens, which lowers the level of catechol estrogens that can be converted to estrogen quinones (21). In turn, the estrogen quinones are expected to undergo conjugation with GSH either nonenzymatically or, more efficiently, via the catalytic action of GSTs, a superfamily of multifunctional Phase II enzymes that play a key role in cellular detoxification (22–25). The formation of GSH-estrogen conjugates would reduce the level of estrogen quinones and thereby lower the potential for DNA damage.

The main support for the genotoxicity of the oxidative estrogen metabolism pathway comes from animal models in which the administration of E2, 2-OHE2, or 4-OHE2 induces cancer (e.g., renal cancer in male Syrian hamsters and endometrial cancer in CD-1 mice; Refs. 11, 26, and 27). However, direct experimental evidence for each step of the pathway and the underlying mechanism of enzyme kinetics is limited to studies of the CYP1A1- and CYP1B1-mediated conversion of E2 to 2-OHE2 and 4-OHE2 and the catechol-O-methyltransferase-mediated methylation of the latter two compounds to the respective methoxy estrogens (5–7, 21). Several investigators have used hamster or rat liver microsomes to examine the subsequent metabolic conversion of 2-OHE1 and 4-OHE2 to estrogen quinones and GSH-estrogen conjugates, with the inherent limitation of not completely knowing the identity of the enzymes and the reaction involved (28–30). In particular, the interaction of the Phase I and II enzymes (i.e., CYPs and GSTs) has not been elucidated. This interaction is important not only in the oxidative estrogen metabolism pathway but also in the metabolism of many exogenous carcinogens, in which the CYPs typically produce the biologically active compounds whereas the GSTs detoxify by converting the latter to water soluble conjugates that can be excreted (31). Thus, the oxidative estrogen metabolism pathway serves as a paradigm for the interaction of Phase I and II enzymes in carcinogenesis.

Although other Phase I enzymes, such as CYP1A2 and CYP3A4, are involved in hepatic and extrahepatic estrogen oxidation, CYP1A1 and CYP1B1 display the highest levels of expression in breast tissue (32, 33). In turn, CYP1B1 exceeds CYP1A1 in its catalytic efficiency as E2 hydroxylase and differs from CYP1A1 in its principal site of catalysis (5, 6, 34). CYP1B1 has its primary activity at the C-4 position of E2, whereas CYP1A1 has its primary activity at the C-2 position in preference to 4-hydroxylation. The 4-hydroxylation activity of CYP1B1 has received particular attention because of experimental evidence that 4-OH catechol estrogens are more carcinogenic than the 2-OH isomers. Treatment with 4-OHE2, but not 2-OHE2, induced renal cancer in the Syrian hamster (11, 26). Analysis of renal
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DNA demonstrated that 4-OHE2 significantly increased 8-hydroxyguanosine levels, whereas 2-OHE2 did not cause oxidative DNA damage (16). Comparison of the corresponding estrogen quinones showed that E2-3,4-Q produced two to three orders of magnitude higher levels of depurinating adducts than E2-2,3-Q (4). In addition to the induction of renal cancer in the hamster model, 4-OHE2 is capable of inducing uterine adenocarcinoma, a hormonally related cancer, in mice. Administration of E2, 2-OHE2, and 4-OHE2 induced endometrial carcinomas in 7, 12, and 66%, respectively, of treated CD-1 mice (27). Finally, examination of microsomal E2 hydroxylation in human breast cancer showed significantly higher 4-OHE2,2-OHE2 ratios in tumor tissue than in adjacent normal breast tissue (35). All of these findings support a causative role of 4-OHE2 in carcinogenesis and implicate CYP1B1 as a key player in the process. GSTs, like the P450 enzymes, are expressed in all organs, with individual family members showing specific patterns of tissue expression. The predominant members in breast tissue is GSTP1 (36). For these reasons, we decided to examine the combined action of CYP1B1 and GSTP1 in oxidative metabolism, using recombinant, purified enzymes. We measured the parent hormone E2, the catechol estrogens, and the GSH conjugates and thereby gained direct insight into mammary estrogen metabolism, an important step for understanding estrogen-induced carcinogenesis.

MATERIALS AND METHODS

Chemicals. E2, 2-OHE2, and 4-OHE2 were obtained from Steraloids (Newport, RI). Deuterated E2, -4-, -16-, and -16-d4 (E2-d4) and 4-OHE2, -1-, -2-, -16-, and -17-d5 (4-OHE2-d5) were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada).

Synthesis of GSH-Estrogen Quinone Conjugates. Conjugates were synthesized by first preparing the estrogen quinone (37, 38), followed by the reaction with GSH (39). For example, 4-OHE2-2-SG was obtained by adding 30 mg (330 µmol) of activated MnO2 to 10 mg (35 µmol) of 4-OHE2 in 10 ml of tetrahydrofuran at 4 °C under nitrogen. After 10 min, the suspension was passed through a glass syringe equipped with two filters (2.0 and 0.45 µm) in tandem to remove MnO2. The yellow filtrate was added dropwise to a solution of 30 mg (120 µmol) of reduced GSH in 1.5 ml of acetic acid:water (1:1, v/v). The solution was stirred at room temperature for 5 min, and aliquots were stored at −20 °C until purification. We used 4-OHE2-d4 in a similar fashion to prepare 4-OHE2-2-SG for use as an internal standard. Note that one deuterium atom is lost during the conjugation step. Each of the products was purified by HPLC on a 4.6 × 150-mm Zorbax XDB 5-µm C8 column with a methanol-water-acetic acid gradient system using an Agilent 1100 Series HPLC (Palo Alto, CA) by monitoring the peak absorbance at 295 nm. Fractions were combined, evaporated to dryness, and weighed. Stock solutions were prepared from the weighed standards and dissolved in methanol to a concentration of 1.58–3.20 µM and stored at −20 °C. The purified products, thus, obtained were 2-OHE2-1-SG, 4-OHE2-2-SG, and 4-OHE2-2-SG-d1, 16,16,17-d4 (4-OHE2-2-SG-d4). Under these conjugation conditions, 2-OHE2-4-SG was a minor product that could not be purified sufficiently to be used as a quantitative standard. Isotopic enrichment of 4-OHE2-2-SG was determined by electrospray ionization using a TSQ Quantum triple quadrupole LC/MS (Thermo Finnigan, Austin, TX) operating with an ion peak width of 0.2 a.m.u. (m/DM = 3000).

Expression and Purification of Recombinant Enzymes. Purified recombinant CYP1B1 and NADPH-P450 reductase were prepared as described previously (7, 40). The breast cancer cell line HS 578T served as source for GSTP1 cDNA. Primers were designed to contain EcoRI and XhoI sites, respectively, at their S' ends to allow amplification of GSTP1 cDNA and ligation of the PCR product into vector pBK-CMV (Stratagene, La Jolla, CA). The ligated vector/insert was transformed into XL1-Blue MRF+ and colonies harboring the correct sequence (verified by restriction digest and complete DNA sequencing) were selected to express GSTP1 protein. Bacterial cultures were grown overnight in Terrific Broth (Life Technologies, Gaithersburg, MD) and induced with 1 mm isopropyl-β-thiogalactopyranoside for 2 h. Cells were harvested by centrifugation at 5,000 × g for 20 min, and spheroplasts were prepared by exposure to lysozyme. The spheroplasts were disrupted by nitrogen in 100 mM Tris-HCl (pH 7.4), 0.3 mM NaCl, and 10 µM each of aprotinin, leupeptin, and pepstatin. The pellet obtained after centrifugation at 10,000 × g for 20 min was discarded, and the supernatant was centrifuged overnight at 110,000 × g. The resultant supernatant was applied to a preequilibrated GSH affinity column (41). The column was washed with at least 50 column volumes of 100 mM Tris-HCl (pH 7.4) and 0.3 mM NaCl. GSTP1 protein was eluted with two column volumes of 100 mM Tris-HCl (pH 7.8), 0.3 mM NaCl, and 10 mM GSH, and the eluate was dialyzed against 100 mM Tris-HCl (pH 7.4) and 0.3 mM NaCl. SDS-PAGE and silver staining revealed the presence of other proteins, which accounted for about 30% of the total protein concentration (36, 42). Application of GSTP1 to S-hexylglutathione affinity matrix did not improve purification. Therefore, we expressed GSTP1 with a GSH-tag from the plasmid pQE30 (Qiagen, Valencia, CA) and purified it by nickel agarose affinity chromatography as described for other GSTs (43, 44). Silver staining showed GSTP1 migrating as a single band migrating at Mr, 23,000 accounting for over 95% of total protein (data not shown). The GST enzyme activity of the His-tagged protein was indistinguishable from the GSH-purified protein using 1-chloro-2,4-dinitrobenzene as substrate (45).

Assay of Enzyme Activities. Recombinant CYP1B1 (250 pmol), NADPH-P450 reductase (500 pmol), and GSTP1 (1 nmol) were mixed with 60 µg of t-α-dilauroyl-sn-glycero-3-phosphocholine in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 25 µM E2, 2-OHE2 or 4-OHE2, 5 mM glucose 6-phosphate, and 1 mM ascorbate. Reactions were initiated by adding 100 µM GSH, glucose-6-phosphate dehydrogenase (0.5 u/ml), and NADP+ to a final concentration of 0.5 mM. Reactions proceeded for 60 min with gentle shaking at 37 °C. At 0, 2, 5, 10, 20, 40, and 60 min, 400-µl aliquots were transferred to 2 ml of chilled CH2Cl2 for E2 and catechol estrogen analysis, and 50-µl aliquots were transferred to 250 µl of chilled acetonitrile to GSH conjugate analysis.

Gas Chromatography/Mass Spectrometry Analysis of E2 and Catechol Estrogens. E2-d4 was added as an internal standard, and all steroids were extracted into CH2Cl2 by vortex mixing for 30 s. Samples were analyzed as described previously (7).

LC/MS Analysis of Estrogen-GSH Conjugates. 4-OHE2-d4,2-SG (269.4 pmol) was added to each timed aliquot as an internal standard, and samples were taken to dryness in a centrifugal concentrator and then reconstituted in methanol/water (50:50, v/v). Just before analysis, 50 µl of each sample were diluted with 250 µl of 500 µM ascorbic acid in aqueous methanol to prevent oxidation of the GSH adducts to various quinone products. Calibration curves were prepared that covered the range of interest by using the concentrated stock solutions described above. Tentam mass spectrometric analyses were performed by electrospray ionization using a ThermoFinnigan TQ 7000 triple quadrupole instrument operating in the selected reaction monitoring mode. The mass transitions 594 → 319 at 26 eV (2- or 4-OHE2-2-SG) and 598 → 322 at 26 eV (4-OHE2-d4,2-SG) were used with an ion integration time of 500 ms/ion. Nitrogen was used as the nebulization and auxiliary drying gas, the spray voltage was 5.0 kV, and the capillary lens was heated to 230°C. The collision cell was pressurized to 2.5 mTorr with argon. HPLC separations were performed in a Waters Alliance 2695 LC with a 2.0 × 250-mm YMC ODS-AQ 5-µm, 120-Å column (Waters, Franklin, MA) using an acetonic-water-acetic acid gradient system. Solvent A was acetonic-water:acetic acid (5:95:0.5), and solvent B was acetonic-water:acetic acid (5:95:0.5). The gradient was programmed at 0.200 ml/min from 0% B (isocratic for 2 min), then decreased linearly to 90% B until 20 min. At the end of each run, the column was washed using 90% B for 4 min to remove hydrophobic steroids and detergents.

RESULTS

We used gas chromatography-mass spectroscopy to quantitate E2, 2-OHE2, and 4-OHE2 and LC/MS to quantitate 2-OHE2-1-SG, 4-OHE2-4-SG, and 4-OHE2-2-SG. To accomplish the LC/MS quantitation, we synthesized the GSH conjugates of 2-OHE2 and 4-OHE2, as well as 4-OHE2-d4, as an internal standard. The conjugates were purified by HPLC and then analyzed by tandem LC/MS. The tandem
MS fragmentation patterns were identical to those reported by Ramanaathan et al. (46). The isotope distribution (Fig. 1) of the purified internal standard 4-OHE2-2-SG-d4 was 0.74% d0, 1.88% d1, 6.15% d2, 6.83% d3, and 84.6% d4. No ions were sufficiently unique among the first-order collision products to be able to distinguish among the isomers. However, the chromatographic conditions reported here gave baseline separation of the three isomers targeted for quantitative analysis (Fig. 2) with an elution order identical to that reported by Cao et al. (30). During development, we observed formation of significant levels of oxidized forms of the various GSH adducts that eluted earlier than the parent metabolite and had a protonated ion at m/z 592. These appeared to be quinone products that formed on standing in air. The addition of 500 μM ascorbic acid to the diluent prevented formation of this byproduct and improved reliability of the assay. Calibration curves were linear over the range 0.015–9.5 μM (r^2 = 0.995).

The rate of nonenzymatic GSH conjugation of catechol estrogens over 60 min was found to be negligible, as determined by incubation of 2-OHE2 and 4-OHE2 with GSH alone (Figs. 3, A and B, and 4A). The addition of the enzyme GSTP1 did not noticeably increase the conjugation (Figs. 3, A and B, and 4A). In contrast, the addition of CYP1B1 significantly enhanced the formation of conjugates, the production of which increased in a nearly linear fashion during the 60-min reaction (Figs. 3, A and B, and 4A). When both CYP1B1 and GSTP1 were added, the rate of conjugation was accelerated with a hyperbolic pattern of product formation (Figs. 3, A and B, and 4A). These results support the theory that the CYP1B1-mediated formation of estrogen quinones is necessary for GSH conjugation. Once the quinones are formed, conjugation can occur nonenzymatically, but the reaction rate is fastest under the combined action of CYP1B1 and GSTP1. Although 2-OHE2 and 4-OHE2 behaved in a similar manner in the four types of reaction, there were distinct differences. In the presence of CYP1B1 and GSTP1, 2-OHE2 yielded two mono-conjugates (i.e., 2-OHE2-1-SG and 2-OHE2-4-SG; Fig. 3, A and B), whereas 4-OHE2 yielded only one mono-conjugate (i.e., 4-OHE2-2-SG; Fig. 4A). Examination of the full spectrum did not reveal the presence of bis-conjugates such as 2-OHE2-1,4-bis-SG and 4-OHE2-1,2-bis-SG. The amount of conjugate produced by CYP1B1 and GSTP1 was highest for 4-OHE2-2-SG, followed by 2-OHE2-4-SG and 2-OHE2-1-SG, the latter accounting for <one-tenth of the two former products. Interestingly, although the nonenzymatic formation of 2-OHE2-1-SG proceeded slower than the GSTP1-mediated production, over time it exceeded the enzymatically formed conjugate, indicating that 2-OHE2-1-SG formation is primarily attributable to spontaneous reaction with GSH (Fig. 3B). Another difference became apparent on monitoring the use of 2-OHE2 and 4-OHE2 (Figs. 3C and 4B). In the presence of GSH and CYP1B1, 2-OHE2 declined in an exponential manner, whereas 4-OHE2 declined in a linear fashion. On combination of GSH, CYP1B1, and GSTP1, both catechol estrogens declined exponentially but 2-OHE2 was completely metabolized by 30 min, whereas one-third of 4-OHE2 remained unmetabolized at 60 min.

Because CYP1B1 readily oxidizes E2 to 2-OHE2 and 4-OHE2 and our present results showed expedient conversion of the latter coupling to GSH-estrogen conjugates by CYP1B1 and GSTP1, we decided to examine the entire sequence of reactions starting with the parent hormone E2 as substrate. The molar ratio of CYP1B1 to GSTP1 in breast tissue is unknown but likely to vary because the CYP1B1 and GSTP1 genes are inducible by numerous agents. Therefore, we held the amount of CYP1B1 constant at 250 pmol and varied the molar ratio of GSTP1 from 0.5 to 4 (i.e., 125–1000 pmol; Fig. 5). After a 10-min incubation of 25 μM E2 with CYP1B1, we observed a final concentration of 5.5 ± 0.3 μM 2-OHE2 and 7.0 ± 0.3 μM 4-OHE2 when GSTP1 levels ranged from 125 to 750 pmol. Only 1000 pmol of GSTP1 caused a noticeable decrease in the concentration of 2-OHE2 to 3.9 ± 0.2 μM and of 4-OHE2 to 6.0 ± 0.1 μM (Fig. 5A). In contrast, the production of GSH-estrogen conjugates increased steadily in
Finally, we analyzed the time course of the metabolism of 10 \( \mu M \) E\(_2\) to catechol estrogens and GSH-estrogen conjugates during a 60-min reaction with CYP1B1 and GSTP1 (Fig. 7). The parent hormone E\(_2\) was completely metabolized within 40 min, showing the steepest decline in the first 5 min, during which time the catechol estrogens rapidly increased in concentration (Fig. 7A). The concentration of 2-OHE\(_2\) peaked at 5 min, whereas that of 4-OHE\(_2\) continued to rise to a peak at 10 min. Both catechol estrogens then slowly declined during the remainder of the reaction, with 4-OHE\(_2\) levels staying higher than those of 2-OHE\(_2\). Parallel analysis of the GSH-estrogen conjugates showed a continuing increase in concentration with the steepest rise of 4-OHE\(_2\)-2-SG and 2-OHE\(_2\)-4-SG during the first 10 min. The former continued to increase and clearly exceeded the latter by 60 min (Fig. 7B). Overall, the concentration of 4-OHE\(_2\)-2-SG and 2-OHE\(_2\)-4-SG and the concentration of 2-OHE\(_2\)-1-SG were one and two orders of magnitude, respectively, lower than that of the catechol estrogens.

**DISCUSSION**

Estrogen quinones occupy a pivotal position in the oxidative estrogen metabolism pathway. Ideally, estrogen quinone measurement would help quantify their role, but they are highly reactive with short half-lives because of the strained 1,2-diketone functionality inherent in \( \alpha \)-quinones. For example, the \( \alpha \)-quinones formed from 2-OHE\(_1\) and 4-OHE\(_1\) were determined to have half-lives of 42 s and 12 min, respectively, at pH 7.4 and 37\(^\circ\)C (29). In comparison, the \( \alpha \)-quinone formed from the equine catechol estrogen 4-hydroxyequilenin was much more stable with a half-life of 2.3 h, probably because of the adjacent aromatic B ring (48). The labile estrogen quinones readily react with a variety of physiological compounds, ranging from amino proportion to the amount of GSTP1 added (Fig. 5B). However, the concentration of conjugate was one order of magnitude lower (i.e., 2-OHE\(_2\)-4-SG and 4-OHE\(_2\)-2-SG were 0.15 \( \pm \) 0.01 \( \mu M \) and 0.14 \( \pm \) 0.01 \( \mu M \) for 125 pmol of GSTP1 and 0.29 \( \pm \) 0.01 \( \mu M \) and 0.34 \( \pm \) 0.01 \( \mu M \) for 1000 pmol of GSTP1, respectively). The production of 2-OHE\(_2\)-1-SG was even lower, with a maximum level of 0.013 \( \pm \) 0.002 \( \mu M \) at 1000 pmol of GSTP1.

Previous studies of CYP1B1-mediated E\(_2\) oxidation to catechol estrogens have shown first-order rate kinetics for E\(_2\) concentrations \( \leq 20 \mu M \) (7, 47). These studies were performed in the absence of GSTP1 and GSH. In Fig. 6, we show results obtained for the combination of enzymes and substrates, with E\(_2\) varied from 5 to 25 \( \mu M \). During the 10-min reaction, 2-OHE\(_2\) and 4-OHE\(_2\) increased in a linear fashion in proportion to the added E\(_2\), with 4-OHE\(_2\) exceeding 2-OHE\(_2\) (Fig. 6A). The corresponding rate of production for the GSH-estrogen conjugates was one order of magnitude lower and leveled off at higher E\(_2\) concentrations, indicating saturation of GSTP1 with intermediary metabolites (Fig. 6B). The rate of formation of 2-OHE\(_2\)-4-SG and 4-OHE\(_2\)-2-SG during the 10-min reaction was similar and at least 10-fold higher than the production of 2-OHE\(_2\)-1-SG.
ster or rat liver microsomes with horseradish peroxidase or lactoperoxidase in the presence of 0.5 mM H$_2$O$_2$ and catechol estrogens ranging in concentration from 0.31 to 3.5 mM yielded the mono-GSH conjugates but also GSH-di-conjugates 2-OHE$_2$-1,4-bisSG and 4-OHE$_2$-1,2-bisSG (29, 30), which were not detected under the present experimental conditions with much lower concentrations of 2-OHE$_2$ and 4-OHE$_2$ (≤25 μM).

The equine catechol estrogens 4-hydroxyequilin and 4-hydroxy-equilenin were shown to rapidly autoxidize to quinones under physiological conditions, allowing GSH conjugation in the presence of GSTM1 (53). Here, we show that 2-OHE$_2$ and 4-OHE$_2$ behave differently, resulting in negligible production of conjugates in the presence of GSH alone or combined with GSTP1. Our data indicate that the enzymatic conversion of catechol estrogens to estrogen quinones by CYP1B1 is a necessary step for the subsequent GSH conjugation reaction. GSH, the most abundant intracellular nonprotein thiol, is present in most cells at concentrations ranging from 0.1 to 10 mM (54). The body maintains the ratio between oxidized (GS-SG) and reduced (GSH) forms as low as 0.1, facilitating the conjugation of reduced GSH by GSTs (22, 24). In the present study, we used 0.1 mM GSH and observed a faster rate of conjugation in the presence of GSTP1, resulting in higher levels of 2-OHE$_2$-4-SG and 4-OHE$_2$-2-SG than in the absence of the enzyme.

The steroid hormones E$_2$ and E$_1$ are characterized by an aromatic A-ring and the hydroxyl group at C-3. The enzymes involved in estrogen metabolism, starting with CYP1A1 and CYP1B1, act on the A-ring with the principal sites of catalysis at the vicinal carbons, C-2 and C-4. However, the two carbons are not equivalent for the P450 enzymes. It has been shown that CYP1B1 preferentially catalyzes the C-4 rather than the C-2 hydroxylation, whereas CYP1A1 exclusively

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**Fig. 5.** Metabolism of E$_2$ to catechol estrogens (A) and GSH-estrogen conjugates (B) in the presence of increasing GSTP1 concentrations. Each reaction contained 25 μM E$_2$, 100 μM GSH, 250 pmol of CYP1B1, and 125, 250, 500, 750, and 1000 pmol of GSTP1. Reactions proceeded for 10 min at 37°C and were analyzed for the production of metabolites by GC/MS and LC/MS, as described in “Materials and Methods.” Data are represented as the means of two replicate assays; bars, SD.

Mass spectrometric analysis of the GSH-estrogen isomers revealed that the catechol estrogen attachment is at the cysteine moiety of GSH, with the cysteine sulfur binding to an A-ring carbon vicinal to the catechol carbons (i.e., C-1 or C-4 in 2-OHE$_2$ and C-2 in 4-OHE$_2$; Refs. 39 and 46). Abul-Hajj and Cisek (23, 37) proved the regiospecific attachment of GSH at C-2 by using C-1-tritiated E$_1$-3,4-Q and demonstrating that the conjugation did not eliminate tritium. As predicted by these chemical studies, the enzymatic reaction with GSTP1 yielded two mono-GSH conjugates for 2-OHE$_2$ (2-OHE$_2$-1-SG and 4-OHE$_2$-4-SG) but only one mono-conjugate for 4-OHE$_2$ (4-OHE$_2$-2-SG), in line with all other known quinone-GSH conjugates, in which the point of attachment of the -SG moiety is always directly adjacent to an oxygen-bearing carbon (52). Few biochemical studies have examined the GSH conjugation of estrogen metabolites, using rodent hepatic or renal microsomes (28–30). Although these studies were important in characterizing the GSH-estrogen conjugates analytically, they used considerably higher concentrations of estrogen substrates or added enzymes without knowing the identity or concentration of the microsomal enzymes. For example, incubation of ham-

![Image](image_url)

**Fig. 6.** Metabolism of E$_2$ to catechol estrogens (A) and GSH-estrogen conjugates (B) as a function of E$_2$ concentration. Each reaction contained 100 μM GSH, 250 pmol of CYP1B1, 500 pmol of GSTP1, and 5, 10, 15, 20, and 25 μM E$_2$. Reactions proceeded for 10 min at 37°C and were analyzed for the production of metabolites by GC/MS and LC/MS, as described in “Materials and Methods.” Data are represented as the means of two replicate assays; bars, SD.
catalyzes the C-2 hydroxylation of E2 and E1 (6). Thus, 4-OHE2 is the main product of CYP1B1-mediated oxidation of E2, as demonstrated in the experiments shown in Figs. 5–7. Furthermore, analysis of the second CYP1B1-mediated reaction, in which 4-OHE2 and 2-OHE2 are the substrates, also revealed a difference in reactivity, with 2-OHE2 reacting more quickly than 4-OHE2 (Figs. 3C and 4B). The observed difference in the disappearance of 2-OHE2 and 4-OHE2 may be attributed to the difference in half-lives of the respective products, E2-2,3-Q and E2-3,4-Q, as suggested by the corresponding half-lives of 42 s and 12 min reported for E2-2,3-Q and E2-3,4-Q, respectively (29). One question about the subsequent conjugation reaction was whether GSTP1 would also display site preference for the respective quinones, E2-2,3-Q and E2-3,4-Q. Although the concentration of these quinones could not be measured, GSTP1 does not seem to distinguish between C-2 and C-4 conjugation, because the results in Figs. 5B and 6B showed similar rates of formation for both 4-OHE2-2-SG and 2-OHE2-4-SG. However, the enzyme favored both sites over C-1, with 2-OHE2-1-SG being formed one order of magnitude less than the C-2 and C-4 conjugates. A second question about the GSTP1-mediated reaction was whether it was more or less efficient than the preceding CYP1B1-mediated reaction. In the former case, a highly efficient conjugation reaction would be expected to prevent any build-up of catechol estrogens. Instead, the results in Fig. 7A clearly show the accumulation of 4-OHE2 and 2-OHE2 as intermediary products. However, the persistence of the catechol estrogens could also be caused by gradual inactivation of CYP1B1 and GSTP1 resulting from the nonenzymatic reaction with the labile quinones. It has been shown that incubation of high concentrations (0.3–1.5 mM) of 4-hydroxyequilenin with GSTM1 in the absence of oxidative enzymes resulted in irreversible inactivation of GSTM1, most likely because of covalent modification and/or oxidation of critical amino acid residues (53). A third question was whether we would be able to account, in quantitative terms, for the overall oxidative E2 metabolism. Starting with 10 μM E2, we showed a nearly quantitative conversion to the catechol estrogens (Fig. 7A). Although we expected a somewhat lower yield for the GSH-estrogen conjugates based on their lower position in the metabolic pathway, we were surprised that the concentration was one order of magnitude lower. Again, the most likely explanation for the discrepancy is the interaction of the highly reactive intermediary estrogen quinones with all nucleophiles in the reaction mixture, including GSH, CYP1B1, and GSTP1, leading to the formation of alternate products not measured in our assay. A fourth question was whether other enzymes participate in the GSH conjugation of mammary estrogen metabolites. Besides GSTP1, at least two other members of the GST family are expressed in breast tissue, namely GSTA1 and GSTM1 (36, 55, 56). GSTs are known to have selective as well as overlapping substrate specificities. Although not examined here, it is likely that GSTP1, GSTA1, and GSTM1 share in the conjugation reaction in a similar way as CYP1B1 and CYP1A1 join in the hydroxylation of E2. However, GSTM1 and GSTA1 are not consistently expressed in breast tissue. Approximately 50% Caucasian and 25% African-American women possess the GSTM1 null genotype, and, therefore, lack GSTM1 expression in all tissues including breast (57). In contrast, GSTA1 was either completely absent or present in the lowest percentage of breast cancer cases examined by immunohistochemistry (55, 58). Thus, GSTP1 is the predominant GST catalyzing the GSH conjugation of estrogen quinones in breast tissue with variable contributions by GSTM1 and GSTA1. Like the endogenous estrogens, many exogenous compounds are metabolized by Phase I enzymes, leading to the formation of genotoxic and carcinogenic metabolites, which are further metabolized by Phase II enzymes such as the GSTs. On the basis of the sequential reactions examined here, the present study of CYP1B1 and GSTP1 serves as a paradigm for the
interaction of Phase I and II enzymes in both qualitative and quantitative terms, setting the stage for future studies of the combined contribution of CYP1B1, CYP1A1, GSTP1, GSTM1, and GSTA1 to the metabolism of estrogens and other carcinogens.

In summary, we observed that CYP1B1 and GSTP1 are coordinated in sequential reactions (i.e., 4-OH-E2 and 2-OH-E2 did not form GSH conjugates in the presence of GSTP1 unless they were first oxidized by CYP1B1 to their corresponding quinones). CYP1B1 metabolized E2 to two products, 4-OH-E2 and 2-OH-E2, and further to E2-3,4-Q and E2-2,3-Q, whereas GSTP1 formed three products, 4-OH-E2-2-SG, 2-OH-E2-4-SG, and 2-OHE2-1-SG, the last one in smallest amounts.

E2-2,3-Q and E2-3,4-Q are products of CYP1B1-mediated reactions and substrates of GSTP1-mediated reactions but also react nonenzymatically with other nucleophiles, as indicated by the concentration gap between catechol estrogens and GSH-estrogen conjugates. Although both reactions are coordinated qualitatively in terms of product formation and substrate utilization, the quantitative gap would leave room for the accumulation of estrogen quinones and their potential for DNA damage.

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REFERENCES


Sequential Action of Phase I and II Enzymes Cytochrome P450 1B1 and Glutathione S-Transferase P1 in Mammary Estrogen Metabolism
