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-OHE2,1-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-OHE4,4-SG > 2-OHE2,1-SG. Incubation of E2 with CYP1B1 and GSTP1 resulted in the formation of 4-OHE2,2-OHE2, 4-OHE2,2-SG, 2-OHE4,4-SG, and 2-OHE2,1-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 by simultaneous stimulation of cell proliferation and gene expression (3, 4). The pathway starts with the two major estrogens, E1 and E2, 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 deoxynucleosides (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-OHE2 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 E1 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...
DNA demonstrated that 4-OHE₂ significantly increased 8-hydroxyguaninosine 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 deaminating 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 member in breast tissue is GSTP1 (36). For these reasons, we decided to examine the combined action of CYP1B1 and GSTP1 in oxidative estrogen metabolism, using recombinant, purified enzymes. We measured the parent hormone E₂, the catechol estrogens, and the GSH conjugates and thereby gained direct insight into mammatory 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-, -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 first by 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, stirring 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 mM and stored at −20°C. The purified products, thus, obtained were 2-OHE₂-1-SG, 4-OHE₂-2-SG, and 4-OHE₂-2-SG, 4-OHE₂-4-SG-2-SG-d₅. 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-d₅ 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 5′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 competent cells (Stratagene), 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 short sonication in 100 mM Tris-HCl (pH 7.4), 0.3 M NaCl, and 10 mM 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 diazylated 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-hexylgluthionine affinity matrix did not improve purification. Therefore, we expressed GSTP1 with a N-terminal 6×His 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 M₉23,000 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 pmol) 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 μM), 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 CH₃Cl for E₂ and catechol estrogen analysis, and 50-μl aliquots were transferred to 250 μl of chilled acetone for 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 CH₃Cl by vortex mixing for 30 s. Samples were analyzed as described previously (7).

LC/MS Analysis of Estrogen-GSH Conjugates. 4-OHE₂-d₄ (260 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 was 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 0.015–0.9 pmol. Solutions were transferred to 2 ml of chilled CH₃Cl for E₂ and catechol estrogen analysis, and 50-μl aliquots were transferred to 250 μl of chilled acetone for GSH conjugate analysis.

RESULTS

We used gas chromatography-mass spectroscopy to quantitate E₂₂, 2-OHE₂, and 4-OHE₂ and LC/MS to quantitate 2-OHE₂-1-SG, 2-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 LC/MS analysis of estrogen-GSH conjugates, 4-OHE₂-d₄, 4-2-SG (260 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 was 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 0.015–0.9 pmol. Solutions were transferred to 2 ml of chilled CH₃Cl for E₂ and catechol estrogen analysis, and 50-μl aliquots were transferred to 250 μl of chilled acetone for 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 CH₃Cl 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 (264 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 was 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 0.015–0.9 pmol. Solutions were transferred to 2 ml of chilled CH₃Cl for E₂ and catechol estrogen analysis, and 50-μl aliquots were transferred to 250 μl of chilled acetone for GSH conjugate analysis.
MS fragmentation patterns were identical to those reported by Ramanathan et al. (46). The isotope distribution (Fig. 1) of the purified internal standard 4-OHE\(_2\)-2-SG-d\(_4\) was 0.74% d\(_0\), 1.88% d\(_1\), 6.15% d\(_2\), 6.83% d\(_3\), and 84.6% d\(_4\). 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 \(\mu\)M 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 \(\mu\)M (\(r^2 = 0.995\)).

The rate of nonenzymatic GSH conjugation of catechol estrogens over 60 min was found to be negligible, as determined by incubation of 2-OHE\(_2\) and 4-OHE\(_2\) 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-OHE\(_2\) and 4-OHE\(_2\) behaved in a similar manner in the four types of reaction, there were distinct differences. In the presence of CYP1B1 and GSTP1, 2-OHE\(_2\) yielded two mono-conjugates (i.e., 2-OHE\(_2\)-1-SG and 2-OHE\(_2\)-4-SG; Fig. 3, A and B), whereas 4-OHE\(_2\) yielded only one mono-conjugate (i.e., 4-OHE\(_2\)-2-SG; Fig. 4A). Examination of the full spectrum did not reveal the presence of bis-conjugates such as 2-OHE\(_2\)-1,4-bis-SG and 4-OHE\(_2\)-1,2-bis-SG. The amount of conjugate produced by CYP1B1 and GSTP1 was highest for 4-OHE\(_2\)-2-SG, followed by 2-OHE\(_2\)-4-SG and 2-OHE\(_2\)-1-SG, the latter accounting for <one-tenth of the two former products. Interestingly, although the nonenzymatic formation of 2-OHE\(_2\)-1-SG proceeded slower than the GSTP1-mediated production, over time it exceeded the enzymatically formed conjugate, indicating that 2-OHE\(_2\)-1-SG formation is primarily attributable to spontaneous reaction with GSH (Fig. 3B). Another difference became apparent on monitoring the use of 2-OHE\(_2\) and 4-OHE\(_2\) (Figs. 3C and 4B). In the presence of GSH and CYP1B1, 2-OHE\(_2\) declined in an exponential manner, whereas 4-OHE\(_2\) declined in a linear fashion. On combination of GSH, CYP1B1, and GSTP1, both catechol estrogens declined exponentially but 2-OHE\(_2\) was completely metabolized by 30 min, whereas one-third of 4-OHE\(_2\) remained unmetabolized at 60 min. Because CYP1B1 readily oxidizes E\(_2\) to 2-OHE\(_2\) and 4-OHE\(_2\) and our present results showed expeditious conversion of the latter compounds to GSH-estrogen conjugates by CYP1B1 and GSTP1, we decided to examine the entire sequence of reactions starting with the parent hormone E\(_2\) 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 \(\mu\)M E\(_2\) with CYP1B1, we observed a final concentration of 5.5 \(\pm\) 0.3 \(\mu\)M 2-OHE\(_2\) and 7.0 \(\pm\) 0.3 \(\mu\)M 4-OHE\(_2\); when GSTP1 levels ranged from 125 to 750 pmol. Only 1000 pmol of GSTP1 caused a noticeable decrease in the concentration of 2-OHE\(_2\) to 3.9 \(\pm\) 0.2 \(\mu\)M and of 4-OHE\(_2\) to 6.0 \(\pm\) 0.1 \(\mu\)M (Fig. 5A). In contrast, the production of GSH-estrogen conjugates increased steadily in

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**Fig. 1.** Tandem mass spectrum of the protonated molecular ion of internal standard (4-OHE\(_2\)-2-SG-d\(_4\)) determined with a collision energy of 25 eV and the isotopic distribution (inset) of the precursor ions. The major product ion (\(m/z\) 322) is formed by neutral loss of GSH by cleavage at the glutathionyl side of the sulfur and transfer of deuterium from position 1 of the steroid nucleus to the cysteine.

**Fig. 2.** Selected reaction monitoring chromatographic profile for the GSH conjugates formed by reaction of 10 \(\mu\)M E\(_2\) with 0.5 \(\mu\)M CYP1B1, 1.0 \(\mu\)M NADPH P450 reductase, 1.0 \(\mu\)M GSTP1, and 100 \(\mu\)M GSH (top) and the internal standard (bottom). The chromatographic separations were performed as described in “Materials and Methods.”
Finally, we analyzed the time course of the metabolism of 10 μM E2 to catechol estrogens and GSH-estrogen conjugates during a 60-min reaction with CYP1B1 and GSTP1 (Fig. 7). The parent hormone E2 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-OHE2 peaked at 5 min, whereas that of 4-OHE2 continued to rise to a peak at 10 min. Both catechol estrogens then slowly declined during the remainder of the reaction, with 4-OHE2 levels staying higher than those of 2-OHE2. Parallel analysis of the GSH-estrogen conjugates showed a continuing increase in concentration with the steepest rise of 4-OHE2-2-SG and 2-OHE2-4-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-OHE2-2-SG and 2-OHE2-4-SG and the concentration of 2-OHE2-1-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 o-quinones. For example, the o-quinones formed from 2-OHE1 and 4-OHE1 were determined to have half-lives of 42 s and 12 min, respectively, at pH 7.4 and 37°C (29). In comparison, the o-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-OHE2-4-SG and 4-OHE2-2-SG were 0.15 ± 0.01 μM and 0.14 ± 0.01 μM for 125 pmol of GSTP1 and 0.29 ± 0.01 μM and 0.34 ± 0.01 μM for 1000 pmol of GSTP1, respectively). The production of 2-OHE2-1-SG was even lower, with a maximum level of 0.013 ± 0.002 μM at 1000 pmol of GSTP1.

Previous studies of CYP1B1-mediated E2 oxidation to catechol estrogens have shown first-order rate kinetics for E2 concentrations ≤20 μM (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 E2 varied from 5 to 25 μM. During the 10-min reaction, 2-OHE2 and 4-OHEE2 increased in a linear fashion in proportion to the added E2, with 4-OHE2 exceeding 2-OHE2 (Fig. 6A). The corresponding rate of production for the GSH-estrogen conjugates was one order of magnitude lower and leveled off at higher E2 concentrations, indicating saturation of GSTP1 with intermediary metabolites (Fig. 6B). The rate of formation of 2-OHE2-4-SG and 4-OHE2-2-SG during the 10-min reaction was similar and at least 10-fold higher than the production of 2-OHE2-1-SG.
The equine catechol estrogens 4-hydroxyequilenin and 4-hydroxy-

equilenin were shown to rapidly autoxidize to quinones under phys-

iological conditions, allowing GSH conjugation in the presence of

GSTM1 (53). Here, we show that 2-OHE2 and 4-OHE2 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 qui-

nones 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-OHE2-4-SG and 4-OHE2-2-SG

than in the absence of the enzyme.

The steroid hormones E2 and E1 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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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-OHE2 and C-2 in 4-OHE2; Refs. 39 and 46). Abul-Hajj and Cisek (23, 37) proved the regiospe-
cific attachment of GSH at C-2 by using C-1-tritiated E1-3,4-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-OHE2 (2-OHE2-

1-SG and 4-OHE2-4-SG) but only one mono-conjugate for 4-OHE2

(4-OHE2-2-SG), in line with all other known quinone-GSH conjuga-
gates, 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 concen-

tration of the microsomal enzymes. For example, incubation of ham-

ster or rat liver microsomes with horseradish peroxidase or lactoper-

oxidase in the presence of 0.5 mM H2O2 and catechol estrogens

ranging in concentration from 0.31 to 3.5 mM yielded the mono-GSH

conjugates but also GSH-di-conjugates 2-OHE2-1,4-bisSG and

4-OHE2-1,2-bisSG (29, 30), which were not detected under the pres-

ent experimental conditions with much lower concentrations of

2-OHE2 and 4-OHE2 (≤25 μM).

Fig. 5. Metabolism of E2 to catechol estrogens (A) and GSH-estrogen conjugates (B) in the presence of increasing GSTP1 concentrations. Each reaction contained 25 μM E2, 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.

Fig. 6. Metabolism of E2 to catechol estrogens (A) and GSH-estrogen conjugates (B) in the presence of increasing GSTP1 concentrations. Each reaction contained 25 μM E2, 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.
resulted in irreversible inactivation of GSTM1, most likely because of 4-hydroxyequilenin with GSTM1 in the absence of oxidative enzymes described in °C and were analyzed for the production of metabolites by GC/MS and LC/MS, as at 37 CYP1B1, and 500 pmol of GSTP1. Reactions proceeded for 0, 2, 5, 10, 20, 40, and 60 min quinones, E2-2,3-Q and E2-3,4-Q. Although the concentration of these whether GSTP1 would also display site preference for the respective (29). One question about the subsequent conjugation reaction was with 2-OHE2-1-SG being formed one order of magnitude less than the 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 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 (33). 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

Fig. 7. Metabolism of E2 to catechol estrogens (A) and GSH-estrogen conjugates (B) as a function of time. Each reaction contained 10 μM E2, 100 μM GSH, 250 pmol of CYP1B1, and 500 pmol of GSTP1. Reactions proceeded for 0, 2, 5, 10, 20, 40, and 60 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.

Fig. 8. Pathway of oxidative estrogen metabolism. CYP1B1 catalyzes the oxidation of E2 to catechol estrogens 2-OHE2 and 4-OHE2. The catechol estrogens are further oxidized to semiquinones (E2-2,3-SQ and E2-3,4-SQ) and quinones (E2-2,3-Q and E2-3,4-Q). GSTP1 catalyzes the conjugation of GSH to the estrogen quinones, leading to the formation of 4-OHE2-2-SG, 2-OHE2-4-SG, and 4-OHE2-1-SG. CYP1B1 preferentially forms 4-OHE2-1-SG and GSTP1 favors the formation of 4-OHE2-2-SG and 2-OHE2-4-SG as indicated by the larger arrows.
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-Q, 2-OH-E2-4-Q, and 2-OH-E2-1-Q, 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
