Chem. Res. Toxicol. **2001,** *14,* 1041−1050

Imbalance of Estrogen Homeostasis in Kidney and Liver of Hamsters Treated with Estradiol: Implications for Estrogen-Induced Initiation of Renal Tumors

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Received February 26, 2001

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Reaction of endogenous catechol estrogen quinones (CE-Q) with DNA may initiate cancer by generation of oncogenic mutations. Treatment of male Syrian golden hamsters with estrogens or 4-catechol estrogens (4-CE), but not 2-CE, induces kidney, but not liver, tumors. The hamster provides an excellent model for studying activation and deactivation (protection) of estrogen metabolites in relation to formation of CE-Q. Several factors can unbalance estrogen homeostasis, thereby increasing the oxidative pathway leading to the carcinogenic CE-3,4-Q. Hamsters were injected with 8 μmol of estradiol (E_2), and liver and kidney extracts were analyzed for 31 estrogen metabolites, conjugates, and depurinating DNA adducts by HPLC with electrochemical detection. Neither liver nor kidney contained 4-methoxyCE, presumably due to the known inhibition of catechol-*O*-methyltransferase by 2-CE. More *O*-methylation of 2-CE was observed in the liver and more formation of CE-Q in the kidney. These results suggest less protective methylation of 2-CE and more pronounced oxidation of CE to CE-Q in the kidney. To investigate this further, hamsters were pretreated with L-buthionine(*S*,*R*)-sulfoximine to deplete glutathione levels and then treated with E_2. Compared to the liver, a very low level of CE and methoxyCE was observed in the kidney, suggesting little protective reductase activity. Most importantly, reaction of CE-3,4-Q with DNA to form the depurinating 4-hydroxyE_2(E_1)-1-N7Gua adducts was detected in the kidney, but not in the liver. Therefore, tumor initiation in the kidney appears to arise from relatively poor methylation of 2-CE and poor reductase activity in the kidney, resulting in high levels of CE-Q. Thus, formation of depurinating DNA adducts by CE-3,4-Q may be the first critical event in the initiation of estrogen-induced kidney tumors.

Introduction

Chemical carcinogens covalently bind to DNA to form two types of adducts,[1] stable ones that remain in DNA unless removed by repair and depurinating adducts that are lost from DNA by destabilization of the glycosyl bond (*1*, *2*). Evidence that depurinating polycyclic aromatic hydrocarbon-DNA adducts play a major role in tumor initiation (*1*, *2*) has provided the impetus for discovering the estrogen metabolites that form depurinating DNA adducts and can be potential endogenous initiators of cancer (*3*). Catechol estrogens (CEs)[2] are among the major metabolites of estrone (E_1) and estradiol (E_2). If these metabolites are oxidized to the electrophilic catechol estrogen quinones (CE-Qs), they may react with DNA. Specifically, the carcinogenic 4-CEs (*4*, *5*) are oxidized to CE-3,4-Q, which react with DNA to form depurinating adducts (*3*, *6*). These adducts generate apurinic sites that may lead to oncogenic mutations (*7*−*9*), thereby initiating cancer.

E_1 and E_2 are obtained by aromatization of 4-androstene-3,17-dione and testosterone, respectively, catalyzed by cytochrome P450 (CYP)19 (aromatase, Figure 1). The estrogens E_1 and E_2 are biochemically interconvertible by the enzyme 17β-estradiol dehydrogenase. E_1 and E_2 are metabolized via two major pathways: formation of CE and, to a lesser extent, 16α-hydroxylation (not shown in Figure 1). The CE formed are the 2- and 4-hydroxylated estrogens. In general, these two CEs are inactivated by conjugating reactions, such as glucuronidation and sulfation, especially in the liver (not shown in Figure 1). The most common pathway of conjugation in extrahepatic tissues, however, occurs by *O*-methylation catalyzed by the ubiquitous catechol-*O*-methyltransferase (COMT) (*10*). The presence of CYP1B1 and other 4-hydroxylases could render the 4-CEs, which are usually minor metabolites, as the major ones. Thus, conjugation of 4-CE via methylation in extrahepatic tissues might become insufficient. In that case, competitive catalytic oxidation of CE to CE-Q could occur (Figure 1). Redox cycling generated by reduction of CE-Q to CE semiquinones, catalyzed by CYP reductase, and subsequent oxidation

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[1] Note: In this article we use the term “adduct” for products formed by reaction of CE-Q with DNA and the term “conjugate” for products formed with GSH and its derivatives.

[2] Abbreviations: BSO, L-buthionine(*S*,*R*)sulfoximine; CE, catechol estrogen(s); CE-Q, catechol estrogen quinone(s); COMT, catechol-*O*-methyltransferase; CYP, cytochrome P450; Cys, cysteine; E_1, estrone; E_2, estradiol; GSH, glutathione; NAcCys, *N*-acetylcysteine; OHE_2, hydroxyestradiol.

10.1021/tx010042g CCC: $20.00 © 2001 American Chemical Society
Published on Web 07/13/2001

Figure 1. Scheme showing the activation and/or deactivation of estrogen metabolites, conjugates, and DNA adducts.

back to CE-Q by molecular oxygen forms superoxide anion radicals and, subsequently, hydroxyl radicals (not shown). In turn, hydroxyl radicals can produce lipid hydroperoxides (*11*, *12*), which are unregulated cofactors of CYP, thereby enhancing oxidation of CE to CE-Q.

CE-Q can be neutralized by conjugation with glutathione (GSH), catalyzed by *S*-transferase. The second inactivating process for CE-Qs is their reduction to CE by quinone reductase and/or cytochrome P450 reductase (*13*, *14*).

Nutter et al. found that quinone reductase purified from rat liver did not reduce E_1-3,4-Q, whereas cytochrome P450 reductase did reduce this substrate (*15*). The CE-Qs are relatively stable up to pH 7 (*16*), but above this pH, they can rapidly polymerize. This does not depend only on pH, but can be triggered by compounds in the environment of the quinone. The assays reported by Nutter et al. (*15*) were conducted at pH 7.5–8.0, suggesting that the lack of reduction of E_1-3,4-Q may have occurred because the quinone rapidly polymerized. Therefore, the ability of quinone reductase to catalyze the reduction of CE-Q to CE remains an open question, and we still hypothesize that this enzyme is involved in the reduction of CE-Q.

If the two inactivating processes, namely, conjugation with GSH and enzymic reduction to CE, are insufficient, CE-Q may react with DNA to form stable and depurinating adducts (*17*). The noncarcinogenic 2-CEs (*4*, *5*) are oxidized to form predominantly stable adducts (*16*, *18*), whereas the carcinogenic 4-CEs (*4*, *5*) are oxidized to form predominantly depurinating adducts (*3*, *6*, *16*).

As seen in Figure 1, there are several factors that can unbalance estrogen homeostasis, with the result of increasing the level of the oxidative pathway leading to ultimate carcinogenic CE-3,4-Q. The first critical factor is excessive synthesis of E_2 by overexpression of aromatase in target tissues (*19–21*) and/or the presence of excess sulfatase that converts stored E_1 sulfate to E_1 (*22*). The observation that breast tissue can synthesize E_2 in situ suggests that much more E_2 is present in some locations of target tissues than would be predicted from plasma concentrations. In animal models such as the Syrian golden hamster and ACI rat, implantation of E_2 produces tumors in the kidney (*23*, *24*) and mammary gland (*25*), respectively, presumably due to imbalances in estrogen homeostasis. A second critical factor might be high levels of 4-hydroxyestradiol (4-OHE_2), due to overexpression of CYP1B1, which converts E_2 predominantly to 4-OHE_2 (*26–28*). This could result in accumulation of 4-CEs and subsequent oxidation to their CE-3,4-Q. A third factor could be a lack or low level of COMT activity. If this enzyme is insufficient, either through its low activity allele or a low level of expression, 4-CE will not be effectively methylated, but will be oxidized to the ultimate carcinogenic metabolites CE-3,4-Q. Fourth, a low level of GSH, and/or a low level of quinone reductase and/or cytochrome P450 reductase can leave available a higher level of CE-3,4-Q that may react with DNA.

In the studies reported here, we investigated the effects of two imbalancing factors on estrogen metabolism in Syrian golden hamsters. The first study involved treatment of hamsters with exogenous E_2. The second one

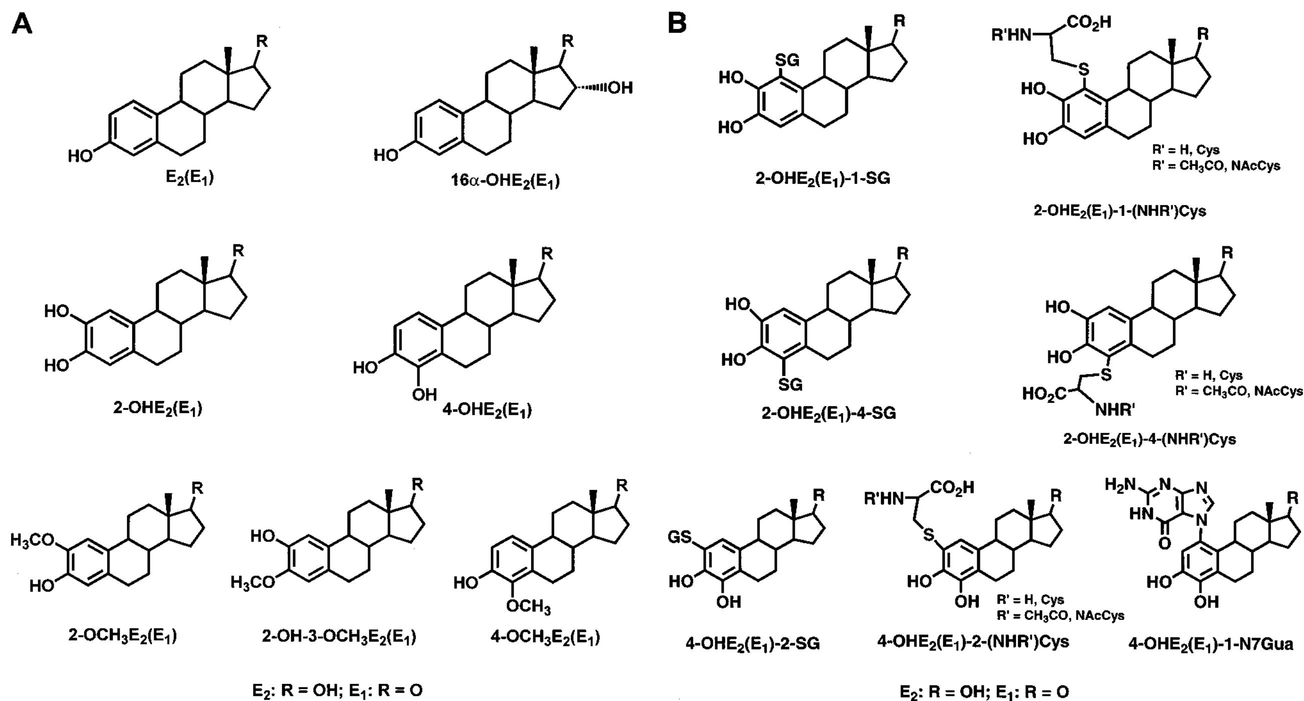

Figure 2. Structures of (A) E_2, E_1, $E_2(E_1)$ metabolites and methoxyCE, and (B) CE-Q conjugates and N7Gua adducts.

examined the effects of E_2 and lowered GSH levels, achieved by treatment of the hamsters with L-buthionine-(*S*,*R*)sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase that is involved in the GSH biosynthesis pathway (*29*, *30*). These studies were conducted in the kidney, which is a good model for estrogen-induced carcinogenesis (*23*, *24*), and the liver, a nontarget tissue.

Materials and Methods

Caution: *E_2 may be a hazardous chemical and was handled carefully in accordance with NIH guidelines (31).*

Materials. Male Syrian golden hamsters (6 weeks old) were from the Eppley Colony. 2-$OHE_1(E_2)$ and 4-$OHE_1(E_2)$ were synthesized according to Dwivedy et al. (*18*). 2-$OHE_1(E_2)$-1-glutathione(−SG), 2-$OHE_1(E_2)$-4-SG, 2-$OHE_1(E_2)$-1-cysteine (Cys), 2-$OHE_1(E_2)$-4-Cys, 2-$OHE_1(E_2)$-1-*N*-acetylcysteine (NAcCys), 2-$OHE_1(E_2)$-4-NAcCys, 4-$OHE_1(E_2)$-2-SG, 4-$OHE_1(E_2)$-2-Cys and 4-$OHE_1(E_2)$-2-NAcCys were synthesized according to published procedures of Cao et al. (*32*). 4-$OHE_1(E_2)$-1-N7Gua were synthesized according to Stack et al. (*16*). Methoxy derivatives of the CE and 16α-OHE_1 were purchased from Steraloids (Newport, R. I.). E_1, E_2, estriol (16α-OHE_2), ascorbic acid, β-glucuronidase (G1512), BSO, and all other enzymes and chemicals were purchased from Sigma (St. Louis, MO). Certify II Sep-Pak cartridges were purchased from Varian (Palo Alto, CA). The Luna(2) C-18 reversed-phase HPLC column (250 × 4.6 mm, 5 μm) was purchased from Phenomenex (Torrance, CA).

Methods. *Treatment of Hamsters.* Groups of four hamsters were treated with E_2. For time-course studies, the animals were intraperitoneally injected with 8 μmol of E_2 dissolved in 300 μL of trioctanoin/DMSO (9:1, v/v) per 100 g of body weight and maintained for 1, 2, or 4 h. For studies with BSO, the animals were subcutaneously injected with 0.6 mmol of BSO in 2 mL of 0.9% saline (per 100 g of body weight) (*29, 30*) 2.5 h before injection of 8 μmol of E_2, dissolved in 300 μL of trioctanoin/DMSO (9:1, per 100 g of body weight) and maintained 2 h after treatment with E_2. Control hamsters were left untreated or treated only with the vehicle and maintained for 2 h. The liver and kidney tissues were collected and stored at −80 °C until use.

Extraction of Metabolites, Conjugates, and Adducts. Kidneys and livers were minced, frozen in liquid nitrogen, and ground to a fine powder. The ground tissue was suspended in 2 mL/g of tissue of 50 mM ammonium acetate, pH 4.4, and homogenized. The homogenate was divided into three portions. One was left untreated, a second was incubated for 6 h at 37 °C, and the third was incubated with β-glucuronidase from *Helix pomatia* (10 000 units, also containing 900 units of arylsulfatase) for 6 h at 37 °C. After incubation, sufficient methanol was added to all fractions to give a final concentration of 60%, and the mixtures were extracted with 8 mL of hexane to remove any lipids. The aqueous phase was diluted with 50 mM ammonium acetate, pH 4.4, containing 1 mg/mL ascorbic acid, to an approximate final concentration of 30% methanol, and the methanol/water mixture was applied to a Certify II Sep-Pak (200 mg) cartridge. The cartridge was first eluted with 3 mL of the buffer, followed by elutions with 2 mL each of 20, 40, and 70% methanol in buffer, and fractions were collected. To minimize oxidation of the CE moieties, ascorbic acid was added to the eluting buffer at a concentration of 1 mg/mL. Collected fractions were analyzed by HPLC with electrochemical detection.

HPLC Analysis. Analyses of the metabolites, conjugates and DNA adducts of E_1 and E_2 (Figure 2) were carried out by using a Luna(2) C18 reversed-phase column (250 × 4.6 mm, 5 μm) on an HPLC system equipped with dual ESA model 580 solvent delivery modules, an ESA model 540 autosampler, and a 12-channel ESA CoulArray electrochemical detector (ESA, Inc., Chelmsford, MA). To analyze metabolites and conjugates, the oxidation potentials were set at −10, 50, 100, 130, 180, 230, 280, 340, 390, 480, 530, and 590 mV, with respect to the internal standard electrode, for channels 1−12. The HPLC column was eluted starting with 78% solvent A [$CH_3CN/CH_3OH/H_2O$/1 M ammonium acetate, pH 4.4 (15:5:70:10)] and 22% solvent B [$CH_3CN/CH_3OH/H_2O$/1 M ammonium acetate, pH 4.4 (50:20:20:10)] for 8.5 min after injection of the sample, followed by a linear gradient to 80% solvent B over 40 min at a flow rate of 1 mL/min. To analyze DNA adducts, the same oxidation potentials were used for channels 1−12, but the column was eluted starting with 100% solvent A [$CH_3CN/CH_3OH/H_2O/CH_3COOH$/1 M sodium acetate, pH 4.4 (10:15:60:5:10)] for 8.5 min after injection of the sample, followed by a linear gradient to 60% solvent B [$CH_3CN/CH_3OH/H_2O/CH_3COOH$/1 M sodium acetate, pH 4.4 (10:55:20:5:10)] over 40 min at a flow rate of 1 mL/min. Metabolites, conjugates, and depurinating DNA adducts from the biological samples were identified by comparison

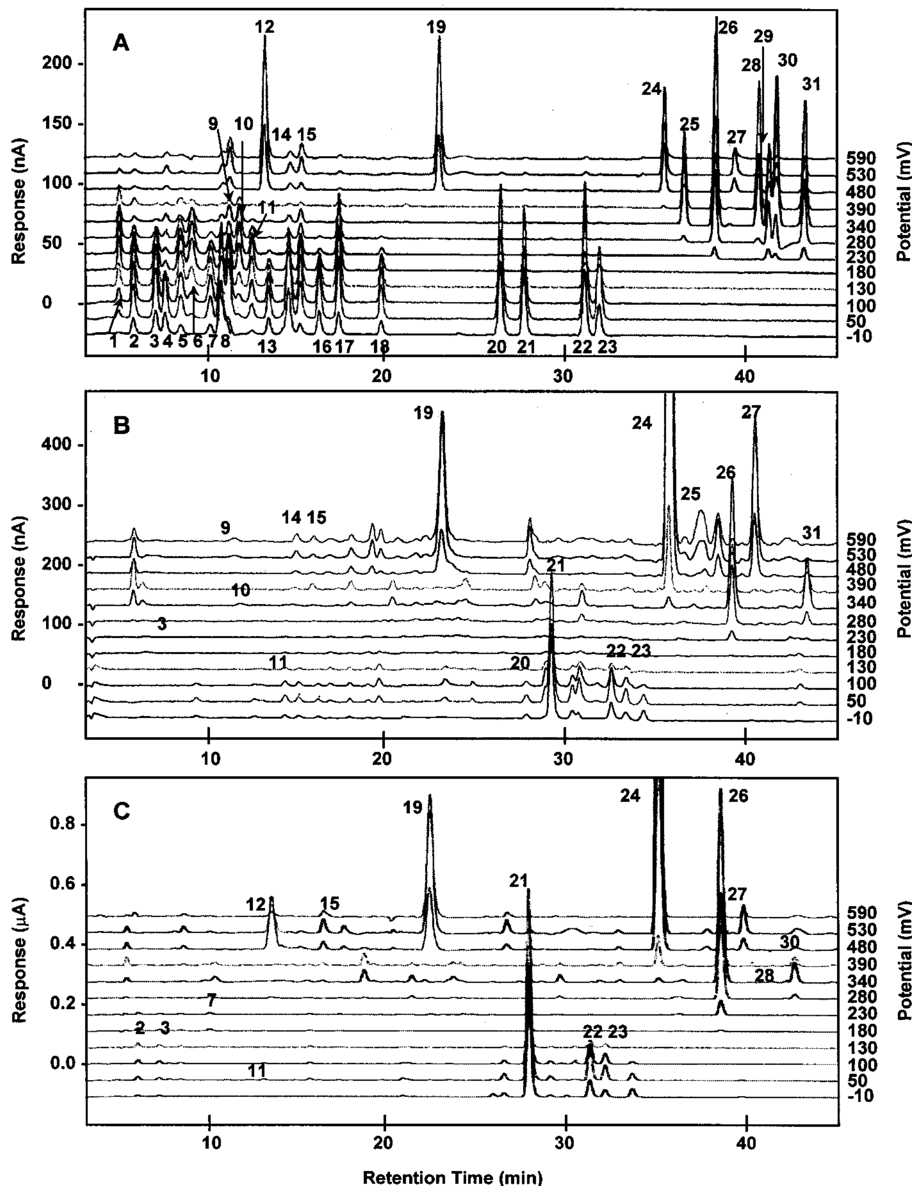

Figure 3. Multichannel electrochemical response from HPLC of (A) standard mixture of estrogens, estrogen metabolites, estrogen conjugates and estrogen-DNA adducts, (B) estrogen compounds detected in the kidney 2 h after treatment of hamsters with E_2, and (C) estrogen compounds detected in the liver 2 h after treatment of hamsters with E_2. The peak numbers correspond to the compounds as follows: **1**, 2-OHE_2-1-SG; **2**, 2-OHE_2-4-SG; **3**, 4-OHE_2-2-SG; **4** & **9**, 2-OHE_2-1(and 4)-Cys; **5**, 2-OHE_1-1(+4)-SG; **6**, 4-OHE_2-1-N7Gua; **7**, 4-OHE_1-2-SG; **8**, 4-OHE_2-2-Cys; **10**, 4-OHE_1-1-N7Gua; **11**, 2-OHE_2-1-NAcCys; **12**, 16α-OHE_2; **13**, 2-OHE_2-4-NAcCys; **14**, 4-OHE_1-2-Cys; **15**, 2-OHE_1-1(+4)-Cys; **16**, 4-OHE_2-2-NAcCys; **17**, 2-OHE_1-1(+4)-NAcCys; **18**, 4-OHE_1-2-NAcCys; **19**, 16α-OHE_1; **20**, 4-OHE_2; **21**, 2-OHE_2; **22**, 2-OHE_1; **23**, 4-OHE_1; **24**, E_2; **25**, 4-OCH_3E_2; **26**, 2-OCH_3E_2; **27**, E_1; **28**, 4-OCH_3E_1; **29**, 2-OH-3-OCH_3E_2; **30**, 2-OCH_3E_1 and **31**, 2-OH-3-OCH_3E_1.

with authentic standards, based on their retention time, as well as the oxidation potential and the peak height ratios between the dominant peak and the peaks in the two adjacent channels (Figure 3A) (*33*). Data analysis was carried out by using ESA CoulArray software. The results presented are the average of two experiments, in which the amount of each analyte differed by 10−30%.

Results

Determination of the ability to maintain estrogen homeostasis, namely the balance between activating and inactivating (protective) metabolism to avoid reaction of CE-Q with DNA, requires identification and quantification of both E_1 and E_2 metabolites, conjugates, and DNA adducts in kidney and liver after treatment of hamsters with E_2. This is necessary because E_1 and E_2 are enzymatically interconvertible. A total of 32 compounds (Figure 2) have been utilized as standards. These include the parent estrogens, E_1 and E_2, the hydroxylated metabolites 16α-$OHE_2(E_1)$, and the CEs 2-$OHE_2(E_1)$ and 4-$OHE_2(E_1)$ (Figure 2A). The methoxyCE conjugates (Figure 2B) can be considered a measure of protection at the CE level. This protection is complemented by glucuronidation and/or sulfation of the hydroxyl groups. The estrogen conjugates with GSH are a measure of CE-Q formation. Their quantification results from the sum of the GSH conjugates plus their products of hydrolysis and acetylation, namely the Cys and NAcCys conjugates, respectively (Figure 2B), which are obtained from the mercapturic acid biosynthesis pathway for the GSH conjugates (*34*). The depurinating 4-$OHE_2(E_1)$-1-N7Gua adducts are used as biomarkers of DNA damage.

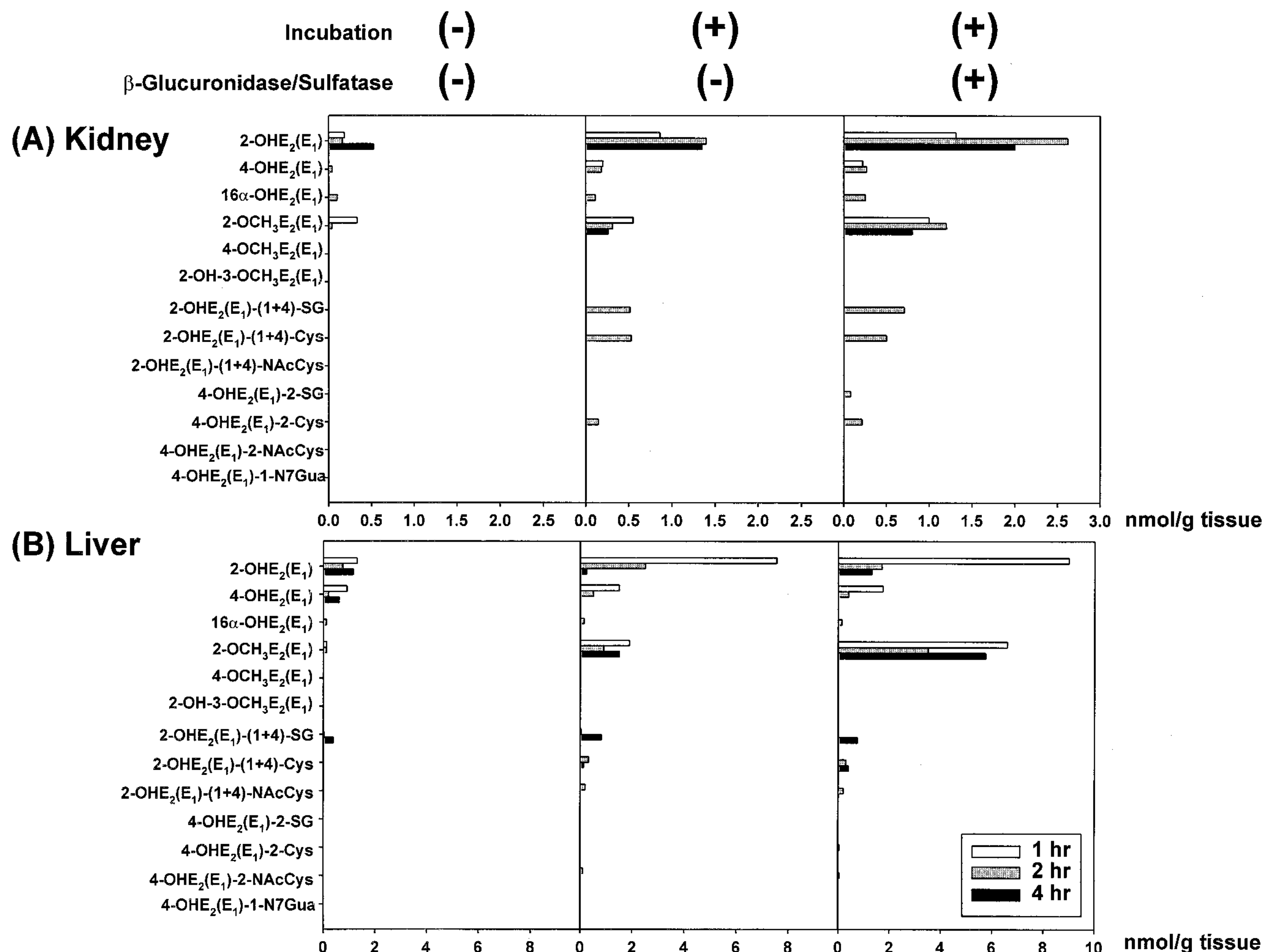

Figure 4. Estrogen compounds detected in the (A) kidney and (B) liver 1, 2, or 4 h after treatment of hamsters with 8 μmol of E_2. The data are the average of two experiments, in which the amount of each analyte differed by 10−30%. Blank spaces indicate that the level of the analyte was less than the limit of detection, 0.01 nmol/g tissue.

Analytical Methods. Analyses of the kidney and liver homogenates were conducted after initial purification on a Certify II Sep-Pak cartridge, which gave >90% recovery of estrogen compounds from tissue samples spiked with estrogen standards (*33*). Separation and identification of estrogen compounds were conducted by HPLC coupled with a 12-channel electrochemical detector that routinely can identify approximately 1 pmol of the estrogen metabolites, conjugates, and adducts. This level of sensitivity is due to the facile electrochemical oxidation of the catechol moiety of most of the analytes. The compounds were identified by comparison with authentic standards, based on their retention time and their oxidation potential. By carefully selecting the potentials for the 12 electrochemical channels, the amount of each of the compounds oxidized in the individual channels was controlled, allowing a unique "fingerprint"-type identification of the compounds of interest, even in the presence of large amounts of other impurities. For example, the GSH conjugates (Figure 3A) are characterized by short retention times, due to their relatively greater polar properties, and very low oxidation potentials. In contrast, the relatively nonpolar E_2 and 4-OCH_3E_2 have relatively long retention times and higher oxidation potentials. With this methodology, previously validated (*33*), it has been possible to identify and quantify 31 estrogen compounds in a single HPLC run.

Time-Response in E_2-Treated Hamsters. To determine the optimum time of analysis for the various estrogen compounds in the kidney and liver, hamsters were injected intraperitoneally with 8 μmol of E_2 and killed after 1, 2, or 4 h. The dose of E_2 was selected based on the results obtained after treatment of hamsters with 4-OHE_2, in which the GSH, Cys, and NAcCys conjugates could be detected between 0.5 and 16 μmol/100 g of body weight (*33*). The dose of 8 μmol was chosen to compensate for injection of E_2, rather than 4-OHE_2. In the kidney (Figure 4A), GSH conjugates were detected only at 2 h, suggesting that at 1 h they were not yet formed and by 4 h they were already eliminated from the kidney. Therefore, 2 h after treatment with E_2 was selected as the best time for the experiments described below. The major metabolites were 2-$OHE_2(E_1)$, which were detected at all three time points. Far less 4-$OHE_2(E_1)$ were observed and these CEs were not even detected at 4 h. 16α-$OHE_2(E_1)$s were measurable only at 2 h. The 2-$OCH_3E_2(E_1)$s were observed at all three time points, whereas no 4-$OCH_3E_2(E_1)$s were detected at any time point.

Analyses were conducted on samples prepared three different ways. The first was carried out on tissue homogenates. The second was done after incubation of the kidney or liver homogenates for 6 h at 37 °C. Under these conditions, some hydrolysis of conjugates catalyzed

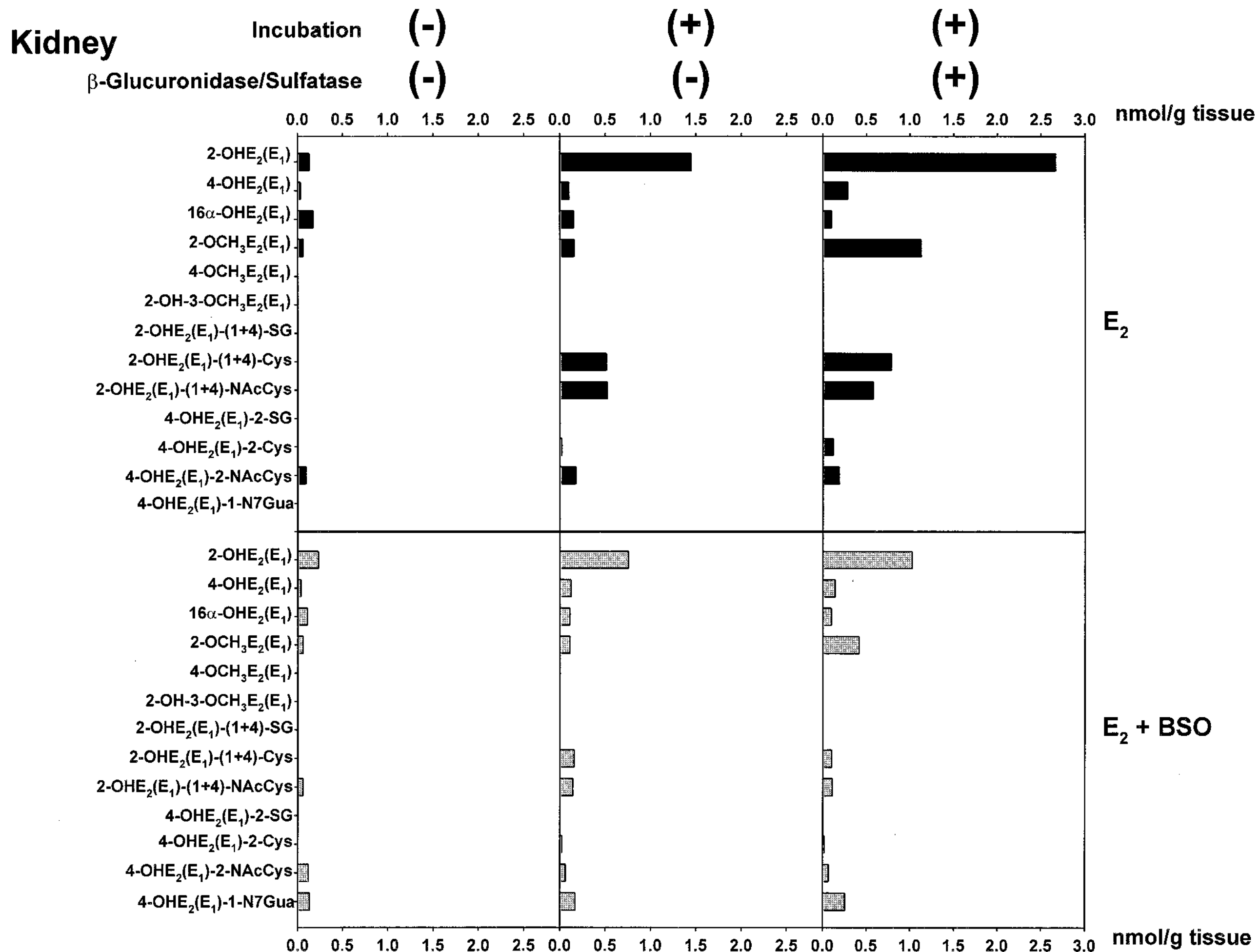

Figure 5. Estrogen compounds detected in the kidney of hamsters treated with 8 μmol of E_2 for 2 h or pretreated with 0.6 mmol of BSO for 2.5 h and then treated with 8 μmol of E_2 for 2 h. The data are the average of two experiments in which the amount of each analyte differed by 10−30%. Blank spaces indicate that the level of analyte was less than the limit of detection, 0.01 nmol/g tissue.

by endogenous enzymes was evident from the increases of some metabolites (Figures 4, 5, and 6). The third was conducted after incubation of the homogenates for 6 h at 37 °C in the presence of added β-glucuronidase/sulfatase (Table 1, Figure 4). Comparison of the unincubated samples with those incubated without or with β-glucuronidase/sulfatase shows a progressive increase of CE and methoxyCE, indicating conjugation by glucuronidation and/or sulfation. Previous results obtained from analysis of urine from hamsters treated with 4-OHE_2 also indicated conjugation of CE and methoxyCE with glucuronic acid (*35*). The 16α-$OHE_2(E_1)$ did not appear to be significantly conjugated (Figures 4 and 5).

Similar results for the CE and methoxyCE were obtained in the liver, including the lack of 4-$OCH_3E_2(E_1)$ and a low amount of 16α-$OHE_2(E_1)$ detectable only at 2 h (Figure 4B). In this organ, relatively low levels of GSH conjugates were obtained, suggesting that less CE is oxidized to CE-Q in the liver than in the kidney. Nevertheless, the GSH conjugates were observed at both 2 and 4 h after treatment with E_2. These results demonstrate that treatment of the hamsters with this dose of E_2 leads to formation of GSH conjugates. The relatively higher levels of GSH conjugates in the kidney (Table 1) suggest that estrogen homeostasis is less balanced in the kidney than in the liver, in that theoxidative pathway leading to formation of CE-Q is favored.

Treatment with E_2 in GSH-Depleted and Nondepleted Hamsters. To confirm that the oxidative pathway of CE to CE-Q is more pronounced in the kidney, a target tissue for estrogen carcinogenesis, than in the refractory liver, hamsters were treated with BSO to deplete GSH levels (*29*, *30*). The dose and time of pretreatment with BSO were selected based on similar studies of GSH depletion conducted in rats (*30*) and hamsters (*29*). In rats, the levels of GSH were 70−80% in the kidney and liver (*30*). After 2.5 h, they were injected with 8 μmol of E_2 for 2 additional hours and then killed. The profiles of estrogen compounds in the liver and kidney were analyzed and compared with those from hamsters treated only with E_2 for 2 h (Figure 5). As expected in this experiment, the hamsters treated with E_2 alone exhibited profiles of estrogen metabolites and conjugates in the kidney and liver that were very similar to those observed in the time-course experiment at 2 h (Figure 4). The predominant metabolites were 2-OHE_2-(E_1), with 10-fold smaller amounts of 4-$OHE_2(E_1)$. Furthermore, 4-$OCH_3E_2(E_1)$ were not detected and 16α-$OHE_2(E_1)$ were low. The amounts of GSH conjugates and their derivatives were relatively more abundant in the kidney than in the liver, suggesting once again a more pronounced oxidative pathway to CE-Q in the kidney.

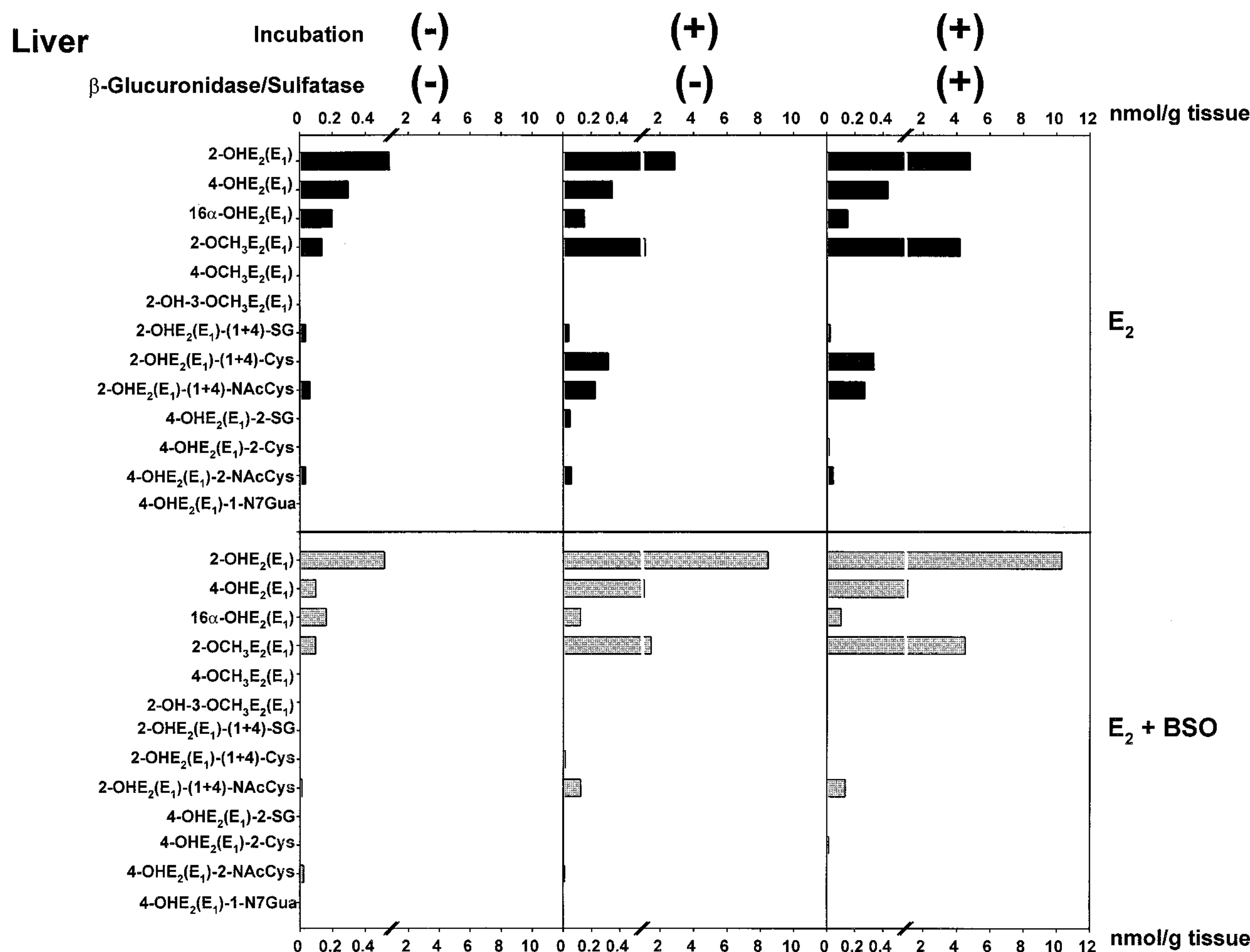

Figure 6. Estrogen compounds detected in the liver of hamsters treated with 8 μmol of E_2 for 2 h or pretreated with 0.6 mmol of BSO for 2.5 h and then treated with 8 μmol of E_2 for 2 h. The data are the average of two experiments in which the amount of each analyte differed by 10–30%. Blank spaces indicate that the level of analyte was less than the limit of detection, 0.01 nmol/g tissue.

When hamsters were pretreated with BSO and treated with E_2, the profile of estrogen compounds in the kidney was characterized by a general decrease in estrogen metabolites and conjugates (Figure 5), whereas in the liver the levels of 2-$OHE_2(E_1)$ and 4-$OHE_2(E_1)$ more than doubled, while the amount of 2-$OCH_3E_2(E_1)$ remained the same (Figure 6). Similarly to the kidney, the levels of GSH conjugates were substantially decreased. Most importantly, reaction of CE-3,4-Q with DNA to form the depurinating 4-$OHE_2(E_1)$-1-N7Gua adducts was detected in the kidney of BSO-pretreated hamsters, but not in the liver. This finding corroborates the oxidative pathway to CE-Q being substantially more pronounced in the kidney than in the liver.

Discussion

Estrogen homeostasis can be defined as the balance between activating and deactivating pathways with the scope of averting the reaction of endogenous CE-Q with DNA. Hypothetically, the balance should be achieved by conjugation of the CE, with only sporadic formation of CE-Q via the oxidative pathway. If the oxidative pathway to CE-Q occasionally occurs, protective conjugation with GSH, catalyzed by GSH-transferases, and/or reduction of the CE-Q, mediated by reductase enzymes that reduce CE-Q back to CE, should prevent reaction of CE-Q with DNA. However, if the oxidative pathway to CE-Q becomes a persistent event and the level of the protective enzymes is insufficient, the CE-Q may react with DNA and produce mutations leading to cancer.

When we disrupt the balance between activation and deactivation by treatment of hamsters with E_2, both the refractory liver and the target kidney produce high levels of 2-CE (90%) compared to 4-CE (10%) (Table 2). In both the kidney and liver, the levels of CE increase upon incubation of extracts at 37 °C for 6 h, and addition of β-glucuronidase/sulfatase enhances their levels even further (Figure 5), suggesting that the phenolic groups are conjugated with glucuronic or sulfuric acid. The same is true for the 2-methoxyCE. In contrast, the unchanging levels of 16α-$OHE_2(E_1)$ suggest that no significant conjugation occurred (Figure 5).

The 2-methoxyCEs are 3.7-fold more abundant in the liver than in the kidney (Table 2), whereas the 4-methoxyCE are not detected in either organ (Table 1, Figure 4). This suggests that the 2-CEs are inhibiting methylation of 4-CEs as previously reported (*36*). Moreover, the total level of CE plus methoxyCE is 2.3-fold higher in the liver than in the kidney. On the contrary, the level of total CE-Q conjugates with GSH, which indicates the level of CE-Q present, is 2.4-fold higher in the kidney (1.36 + 0.30, Table 2) than in the liver (0.63 + 0.06). Furthermore, the level of CE-3,4-Q conjugates is 5-fold higher in the kidney (Table 2). These results suggest that the liver deals much better with conjugation at the CE

Table 1. Estrogen Metabolites and Conjugates Formed in Hamsters Treated with 8 μmol of E_2 for 2 h[a]

metabolite/ conjugate	peak no.[b]	nmol/g tissue					
		not incubated		incubated[c]		incubated[c] with β-glucuronidase/ sulfatase	
		kidney	liver	kidney	liver	kidney	liver
E_2	24	3.80	0.40	2.05	1.17	14.8	17.0
E_1	27	0.57	0.10	1.72	0.61	1.28	0.58
2-OHE_2	21	0.08	0.42	0.82	2.01	1.78	3.05
2-OHE_1	22	0.08	0.32	0.56	0.50	0.84	1.36
4-OHE_2	20		0.11		0.05	0.01	0.07
4-OHE_1	23	0.04	0.07	0.18	0.41	0.26	0.31
16α-OHE_2	12	0.01	0.01	0.08	0.01	0.07	0.03
16α-OHE_1	19		0.10	0.03	0.14	0.23	0.10
2-OCH_3E_2	26	0.04	0.07	0.21	0.34	1.11	2.78
2-OCH_3E_1	30		0.04	0.11	0.54	0.10	0.72
4-OCH_3E_2	25						
4-OCH_3E_1	28						
2-OH-3-OCH_3-E_2	29						
2-OH-3-OCH_3-E_1	31						
2-OHE_2-1-SG	1						
2-OHE_2-4-SG	2						
2-OHE_1-1(+4)-SG	5		0.03		0.03		0.01
2-OHE_2-1(and 4)-Cys	4 & 9			0.30	0.32	0.49	0.29
2-OHE_1-1(+4)-Cys	15			0.22		0.22	
2-OHE_2-1-NAcCys	11						
2-OHE_2-4-NAcCys	13		0.01	0.49	0.15	0.39	0.19
2-OHE_1-1(+4)-NAcCys	17			0.04	0.04	0.11	
4-OHE_2-2-SG	3						
4-OHE_1-2-SG	7				0.02		
4-OHE_2-2-Cys	8					0.03	0.04
4-OHE_1-2-Cys	14					0.05	
4-OHE_2-2-NAcCys	16			0.14		0.21	0.06
4-OHE_1-2-NAcCys	18				0.05		
4-OHE_2-1-N7Gua	6						
4-OHE_1-1-N7Gua	10						

[a] The data presented are the average of two experiments, in which the amount of each analyte differed by 10−30%. Blank spaces indicate that the levels of the analytes were less than the limit of detection, 0.01 nmol/g tissue. [b] Refer to Figure 3 for peak identification [c] Incubation was carried out for 6 h at 37 °C.

Table 2. Critical Estrogen Metabolites, Conjugates, and Adducts Formed in Hamsters Treated with E_2 or E_2 Plus BSO[a]

metabolites/conjugates[b]/ adducts	nmol/g tissue			
	kidney		liver	
	E_2	E_2 + BSO	E_2	E_2 + BSO
2-OHE_2(E_1)	2.66	1.02	4.75	10.27
4-OHE_2(E_1)	0.29	0.14	0.44	1.04
2-OCH_3E_2(E_1)	1.13	0.42	4.16	4.46
E_2(E_1)-2,3-Q conjugates[b]	1.36	0.21	0.63	0.13
E_2(E_1)-3,4-Q conjugates[b]	0.30	0.09	0.06	0.01
E_2(E_1)-3,4-Q N7Gua adducts	$<$0.01	0.27	$<$0.01	$<$0.01

[a] The data reported in this table are from Figure 5, and the tissue extracts were incubated with β-glucuronidase/sulfatase. The limit of detection for each analyte was 0.01 nmol/g tissue. [b] Conjugates include all compounds produced by reaction of CE-Q with GSH and detected with a -SG, -Cys, or -NAcCys moiety.

level than the kidney does, either by methylation, glucuronidation or sulfation, whereas the kidney produces much more CE-Q, implying that the oxidative pathway of CE to CE-Q is more pronounced in the kidney than in the liver and/or reduction of CE-Q to CE catalyzed by quinone reductases is less effective in the kidney (see below).

To investigate whether this interpretation is correct, hamsters were pretreated with BSO to deplete GSH levels and then treated with E_2 for 2 h. In this case, the levels of GSH conjugates were 80% lower in both the kidney and liver, compared to levels in hamsters treated only with E_2, and the depurinating 4-OHE_2(E_1)-1-N7Gua adducts were observed in the kidney (Figure 5), but not in the liver (Figure 6). Furthermore, the levels of CE and methoxyCE were 2.6-fold lower in the kidney of hamsters treated with BSO plus E_2 (1.58, Table 2), compared to those treated only with E_2 (4.18). In contrast, the levels of CE and methoxyCE were 1.7-fold higher in the liver of hamsters treated with BSO plus E_2 (15.77) compared to those treated with E_2 alone (9.35). These results suggest that the effects of GSH depletion are partially offset in the liver by the activity of quinone reductases, whereas the kidney may have very little of this enzymatic activity.

Therefore, when hamsters are treated with E_2 or E_2 plus BSO, the refractory liver copes much better with the attempted imbalance, because of its superior ability to conjugate the CE by methylation, glucuronidation, and sulfation. In addition, with BSO treatment, the diminished protection by GSH, as the conjugating agent for CE-Q, is presumably offset by effective quinone reductase activity that removes the potential danger posed by CE-Q. In the kidney, the presumed lesser capacity to conjugate CE results in more abundant oxidation to CE-Q than in the liver. Furthermore, the apparently ineffective quinone reductases cannot eliminate the potential danger of the CE-Q formed by reducing them to CE. This situation is exacerbated by the low level of GSH after BSO treatment. This major impairment leads to reaction of CE-Q with DNA and formation of the 4-OHE_2(E_1)-1-N7Gua depurinating adducts.

Chronic treatment of hamsters with E_2 leads to kidney tumors, but not liver tumors *(4, 5).* From these studies, it appears that tumor initiation in the kidney occurs by the congruence of two different effects. The relatively poor methylation of CE mediated by COMT leads to more effective oxidation of CE to CE-Q. In addition, the apparently poor quinone reductase activity, as suggested by the results in Table 2, diminishes the conversion of CE-Q to CE. Both effects lead to a large amount of CE-Q, which can react with many biological nucleophiles, including DNA. Formation of depurinating adducts by CE-3,4-Q would thus be the first critical event in the initiation of estrogen-induced kidney tumors. Further experiments to determine the activities of COMT and quinone reductases in the hamster kidney and liver will allow us to demonstrate the roles of these two enzymes in the relative imbalance of estrogen homeostasis.

Acknowledgment. We thank W. Liang for synthesis of 4-OHE_2 and the CE conjugates. This research was supported by U.S. Public Health Service grants P01 CA49210 and R01 CA49917 from the National Cancer Institute. Core support in the Eppley Institute is provided by Grant P30 CA36727 from the National Cancer Institute.

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