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By using NMR and mass spectrometric analyses, we determined the structures of X1 and X2, two representative nonpolar estrogen metabolites, which were metabolically formed following multiple large-scale incubations of 17β-estradiol with human CYP3A4 and NADPH. Both X1 and X2 were unequivocally identified to be the dimers of 17β-estradiol, connected together through a diaryl ether bond between a phenolic oxygen atom of one 17β-estradiol molecule and the 2- or 4-position aromatic carbon of another estrogen. This is the first report for a novel class of the nonpolar 17β-estradiol dimers that are formed from 17β-estradiol by human CYP isoforms in the presence of NADPH as a cofactor.

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Table of Contents

Introduction

The endogenous estrogens undergo extensive metabolism in humans (1, 2), such as oxidation (largely mediated by cytochrome P450 [CYP] enzymes), interconversion between E_2 and E_1, and various conjugation-deconjugation reactions. In addition, the catechol-*O*-methyltransferase (COMT)-mediated *O*-methylation of endogenous catechol estrogens to monomethyl ethers and the acyltransferase-catalyzed esterification of E_2 or hydroxy-E_2 metabolites with various fatty acyl-CoAs result in the production of lipophilic estrogen derivatives that usually have longer half-lives in the body. These multiple metabolic pathways not only determine the pharmacokinetic features of the endogenous estrogens in the body and in various target tissues, but they also diversify the biological actions of endogenous estrogens in certain target sites.

During our recent analysis of the NADPH-dependent oxidative metabolism of [^{3}H]E_2 and [^{3}H]E_1 to various hydroxylated or keto metabolites by human liver microsomes, we consistently detected a cluster of co-eluted radioactive nonpolar peaks (chromatographically less polar than E_1) near the end of a 60-min HPLC run (3, 4). Notably, similar nonpolar radioactive peaks were also noted earlier when [^{3}H]E_2 or [^{3}H]E_1 was incubated with rat or mouse liver microsomes (5-9). However, further characterization of these metabolite peaks has not been pursued mainly because there was no evidence in the literature that would provide any support for the suggestion that nonpolar metabolites could be formed from steroid hormones or xenobiotics by microsomal enzymes using NADPH as a cofactor. However, in our recent study to characterize the NADPH-dependent metabolism of [^{3}H]E_2 and [^{3}H]E_1 by fifteen selectively-expressed human CYP isozymes, we noted that this cluster of nonpolar radioactive peaks appeared to be selectively formed in large amounts only with certain human CYP isoforms, most notably the CYP3A4 and CYP3A5 (10). This interesting observation has prompted us to further investigate the possibility that the formation of some of these nonpolar radioactive E_2 peaks may be catalyzed by CYP isoforms using NADPH as a cofactor. We report here the unequivocal structural identification of two representative nonpolar metabolites formed.

Body

To produce large amounts of X1 and X2 suitable for structural analyses, 200 μM of E_2 was incubated with the selectively-expressed human CYP3A4 (co-expressed with cytochrome b_5) in the presence of an NADPH-generating system in a large scale (60-ml final volume) and using multiple batches of incubations. After the enzymatic reaction, the nonpolar estrogen metabolites X1 and X2 were extracted and then separated with our HPLC system using three different solvent gradients. The first solvent gradient was used to elute all nonpolar estrogen metabolites in the HPLC fraction from 28-37 min (retention time). The second solvent system was used to selectively separate nonpolar estrogen metabolites. X1 and X2 were collected together in the fraction from 43-47 min. The third solvent system was an isocratic elution with 40% acetonitrile, 30% methanol, and 30% water. X1 and X2 were eluted between 33-37 min and 37-40 min, respectively. The third solvent system was used a second time to re-purify X1 and X2. In each separation step, the HPLC trace was monitored with UV detection at 280 nm and the desired fraction was collected from the HPLC column. The acetonitrile and methanol (part of the mobile phase composition) contained in each of the collected HPLC fractions were evaporated first under a mild stream of N_2, and the remaining aqueous solution was then extracted with ethyl acetate to recover the nonpolar metabolites X1 and X2. After multiple steps of HPLC separation, pure X1 and X2 (based on HPLC analysis with UV detection) were obtained.

To identify the structures of X1 and X2, various spectrometric analyses were first performed. UV spectrometric analysis showed that X1 and X2 had the absorption peaks at 281 nm and 278 nm, respectively. Under the same analytical conditions, E_2 and 2-OH-E_2 had absorption peaks at 282 nm and 289 nm, respectively, suggesting that there is only relatively minor modification(s) of the E_2 basic core structure as oppose to the more radical alterations of its structure (such as addition of new chromophore or disruption of the original chromophore). FT-IR spectrometric analysis of X1 and X2 in a dichloromethane solution indicated the absence of the ester or carbonyl group(s) in their structures.

Mass spectrometric analysis provided very useful initial clues about the structures of X1 and X2. Direct probe mass spectrometric analysis showed that both X1 and X2 had a mass of 542 (*m/z*) as their molecular weights. Because the molecular weight of E_2 is 272, a molecular weight of 542 would give a possibility that X1 and X2 may be certain form(s) of the E_2 dimers. Further high-resolution mass spectrometric analysis showed that the molecular weights of X1 and X2 were 542.3400 and 542.3397, respectively. Accordingly, the same chemical formula, $C_{36}H_{46}O_4$ (with its calculated molecular weight of 542.3396), was suggested for both X1 and X2 with only 0.7 and 0.2 *ppm* of error, respectively. The results from high-resolution mass spectrometric analysis greatly increased the possibility that X1 and X2 are E_2 dimers: $2 \times C_{18}H_{24}O_2$ (E_2) – $2H = C_{36}H_{46}O_4$.

To probe the number of hydroxyl groups present in each of the molecules, a small amount of metabolically-formed X1 or X2 (after isolation by using HPLC) were incubated with BSTFA at 65°C for 30 min to convert them into their corresponding TMS derivatives. It is known that addition of each TMS group would increase the molecular weight by 72. Both compounds after TMS derivatization showed a molecular weight mass (*m/z*) of 758 (which equals to 542 + 72 × 3), thereby indicating the presence of three hydroxyl groups in each of the compounds. To identify whether these hydroxyl groups are attached at aliphatic or aromatic carbons, benzyl ether derivatives of X1 and X2 were prepared by their reactions in an acetonitrile solution with benzyl bromide in the presence of potassium carbonate. It is known that only the phenolic hydroxyl group (but not the aliphatic hydroxyl group) can be benzylated by reaction with benzyl bromide under the alkaline conditions devised. The mass spectrometric analyses of the benzyl ether derivatives of both X1 and X2 showed a mass (*m/z*) of 632 as their molecular weight peaks, indicating the presence of only one phenolic hydroxyl group in either X1 or X2. Taken into consideration all the information from spectrometric analyses and chemical derivatization experiments, it seems quite certain that X1 and X2 are the dimers of E_2, with an aryl-aryl ether bond between the C3-position oxygen of one E_2 molecule and an aromatic carbon (at 1 or 2 or 4-position) of another E_2 molecule.

Final definitive evidence for the confirmation of X1 and X2 as E_2 dimers came from various NMR analyses, which include the 1D ^1H, 1D ^{13}C, gDQCOSY, gHMQC, and gHMBC. Experimentally, various NMR spectra of E_2 were obtained first and all proton and carbon peaks were correctly assigned to the appropriate atoms. The ^1H (full scale) and HMQC (aromatic region) NMR spectra for X1 and X2 were then obtained. Overall, the ^1H NMR spectra of X1 and X2 are quite similar to that of E_2 except for the aromatic region (δ 6~8). Notably, because of the very small amounts of metabolically-formed X1 and X2 available, a few additional peaks coming from impurities and solvents were also noted in the NMR spectra. However, these additional peaks could be rationally excluded by analyzing their 2-dimensional NMR spectra. X1 showed two doublets, two singlets, and one doublet of doublet centered at 6.568(d, 2.3 Hz), 6.613(s), 6.641(dd, 8.5 and 2.3 Hz), 6.775(s), and 7.182(d, 8.5 Hz) *ppm* (δ) in the region of the aromatic protons, with equal areas, thus indicating the existence of five aromatic hydrogens in the molecule. Combined with the results of mass spectrometric analysis, the structure of X1 was suggested to be a dimer of E_2, linked together through an aryl-aryl ether bond between the C-3 oxygen atom of one E_2 molecule and the 2-position carbon of another E_2 molecule (**Fig. 1**). The NMR coupling constant of the aromatic protons were assigned as following: 8.5 Hz for $^3J_{1'\text{-}2'}$; 2.3 Hz for $^4J_{2'\text{-}4'}$. This structure was further verified through analysis of the HMBC spectra for its aromatic hydrogens: H-1 correlated with C-2, 3, 5 and 9; H-4 correlated with C-2, 3, 6 and 10; H-1' correlated with C-3', 5' and 9'; H-2' correlated with C-3', 4', 5' and 10'; and H-4' correlated with C-2', 6' and 10'.

Similarly, the proton NMR of X2 showed four doublets and one doublet of doublet centered at 6.461(d, 2.5 Hz), 6.520(dd, 8.5 and 2.5 Hz), 6.732(d, 8.5 Hz), 7.037(d, 8.5 Hz), and 7.136(d, 8.5 Hz) *ppm* (δ) in the region of the aromatic protons, with equal areas, suggesting the existence of five aromatic hydrogens which are different from those of M1. Accordingly, the structure of X2 was also suggested to be a dimer of E_2, linked together through a diaryl ether bond between the C-3 oxygen of one E_2 molecule and the 4-position carbon of another E_2 molecule (**Fig. 1**). This structure was also verified by analyzing the gHMBC spectra for its aromatic hydrogens: H-1 correlated with C-3, 4, 5 and 9; H-2 correlated with C-3, 4 and 10; H-1' correlated with C-3', 5' and 9'; H-2' correlated with C-3', 4' and 10'; H-4' correlated with C-2', 3', 6' and 10'. The final ^1H and ^{13}C NMR resonance assignments of X1 and X2 were made by analyzing the 1D ^1H, 1D ^{13}C, gDQCOSY, gHMQC, and gHMBC datasets.

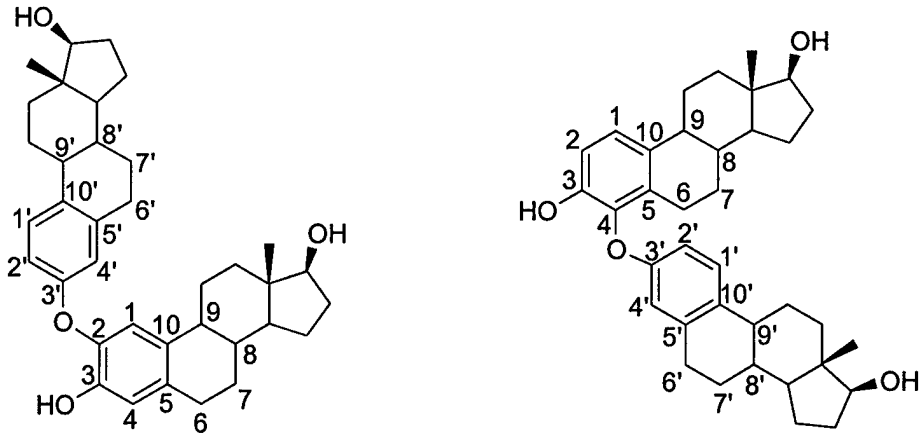

Figure 1. Structures of X1 (left) and X2 (right).

Key Research Accomplishments

- For the first time, we identified the chemical structures of two quantitatively major nonpolar estrogen metabolites, X1 and X2.

Reportable Outcomes

- A patent application (application number not available now).
- One manuscript now in preparation, entitled "Characterization of a Novel Class of Nonpolar 17β-Estradiol Metabolites Formed by Human Cytochrome P450 Enzymes".

Conclusions

We demonstrated, for the first time, that a novel class of nonpolar E_2 metabolites is formed by certain human CYP enzymes. The structures of the metabolically-formed X1 and X2, two representative nonpolar estrogen metabolites, were identified by using various mass and NMR spectrometric analyses. Both X1 and X2 were identified to be the dimers of E_2, which are linked together through an aryl-aryl ether bond between a phenolic oxygen atom of one E_2 molecule and the 2- or 4-position aromatic carbon of another E_2. The potential physiological significance of these metabolites remains to be determined. It will also be of considerable interest to understand the chemical mechanism(s) for the CYP-mediated catalysis leading to the formation of these nonpolar estrogen metabolites.

References

1. Martucci, C. P. & Fishman, J. (1993) *Pharmacol. Ther.* **57**, 237-257.
2. Zhu, B. T. & Conney, A. H. (1998) *Carcinogenesis* **19**, 1-27.
3. Lee, A. J., Kosh, J. W., Conney, A. H. & Zhu, B. T. (2001) *J. Pharmacol. Exp. Ther.* **298**, 420-432.
4. Lee, A. J., Mills, L. H., Kosh, J. W., Conney, A. H. & Zhu, B. T. (2002) *J. Pharmacol. Exp. Ther.* **300**, 838-849.
5. Aoyama, T., Korzekwa, K., Nagata, K., Gillette, J., Gelboin, H. V. & Gonzalez, F. J. (1990) *Endocrinology* **126**, 3101-3106.
6. Suchar, L. A., Chang, R. L., Rosen, R. T., Lech, J. & Conney, A. H. (1995) *J. Pharmacol. Exp. Ther.* **272**, 197-206.
7. Suchar, L. A., Chang, R. L., Thomas, P. E., Rosen, R. T., Lech, J. & Conney, A. H. (1996) *Endocrinology* **137**, 663-676.
8. Zhu, B. T., Lech, J., Rosen, R. T. & Conney, A. H. (1997) *Cancer Res.* **57**, 2419-2427.
9. Zhu, B. T., Loder, D. P., Cai, M. X., Ho, C. T., Huang, M. T. & Conney, A. H. (1998) *Carcinogenesis* **19**, 1821-1827.
10. Lee, A. J., Cai, M. X., Thomas, P. E., Conney, A. H. & Zhu, B. T. (2003) *Endocrinology* in press.