2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN CAUSES AN EXTENSIVE ALTERATION OF ESTRONE METABOLISM IN HUMAN HEPATOCYTE CARCINOMA CELLS: ESTRONE AND ITS SIX METABOLITES ANALYZED BY HPLC-MS/MS

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Abstract
2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is a typical endocrine disruptor. It can bind the aryl hydrocarbon receptor and induce expression of several genes including CYP1A1, CYP1A2, and CYP1B1. Estrogens themselves and their oxidative metabolites are also formed by the activities of various cytochrome P450 enzymes. In the present study, quantitative profiling of estrone (E1) and its metabolites in human Hep G2 cells treated with different amounts of 2,3,7,8-TCDD was reported. E1 levels decreased when the cell culture medium was treated with 2,3,7,8-TCDD. The levels of 2-OH E1, 4-OH E1, 2-OMe E1 and 4-OMe E1 in 2,3,7,8-TCDD-treated cell culture medium were higher than those in untreated cell culture medium and increased with the amount of 2,3,7,8-TCDD added. The levels of 16α-OH E1 in 2,3,7,8-TCDD-treated cell culture medium were lower than that in untreated cell culture medium and decreased with the amount of 2,3,7,8-TCDD added. The quantitative profiling of E1 and its metabolites is considered to provide valuable information for the impacts of the estrogen metabolites on carcinogenesis.

Introduction
Estrogenic hormone is eliminated from the body by metabolic conversions to hormonally inactive or less active water-soluble metabolites that excreted in the urine or feces. In the first stage, transformation of estradiol (E2) into estrone (E1) by oxidation at the C17 position is a reversible process. The reaction normally favors the formation of E1, because E2 proceeds rapidly into E1 while the reverse reduction of E1 to E2 occur considerably more slowly[1]. E1 is further metabolized primarily via oxidative hydroxylation followed by conjugations, including glucuronidation, sulfation, and O-methylation. Estrogens themselves and their oxidative metabolites are formed by the activities of various cytochrome P450 enzymes. These enzymes have dual functions, the biosynthesis and/or inactivation of physiologic regulators on the one hand and the metabolism of environmental chemicals on the other. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is a typical endocrine disruptor. It can bind the aryl hydrocarbon receptor (AhR) and induce expression of several genes including CYP1A1, CYP1A2, and CYP1B1[2]. Animal studies demonstrated that formation of catechol estradiol metabolites (2-OH E2 and 4-OH E2) increased in liver, kidney, and mammary tissues of TCDD-exposed rats[3]. Body burdens of 2,3,7,8-TCDD have been reported to associate with altered ratio of 2-OH E2 to 4-OH E2[4]. The importance of the functional role of estrogen metabolism and the impacts of the estrogen metabolites on carcinogenesis were emphasized in many reviews[5-7]. Therefore, quantitative profiling of estrogens and their metabolites has been considered to provide valuable information. In the present study, quantitative profiling of E1 and its metabolites (Figure 1) in human Hep G2 cells treated with different amounts of 2,3,7,8-TCDD was reported.

Materials and Methods
Human hepatocyte carcinoma cell line, Hep G2 cells (ATCC HB-8065), was grown in a 10-cm dish containing 10 mL of Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco), 10 unit mL-1 penicillin G sodium (Gibco), and 1.5 mg mL-1 sodium bicarbonate (Sigma, St. Louis, MO, USA) under 5% CO2 at 37 °C. At 70-80% confluency, the cells were treated with 10^{-6} M E1 and various amounts of 2,3,7,8-TCDD in DMSO to yield final concentrations of 0, 0.001, 0.01, 0.1, 1.0 and 10.0 nM. After 24 hrs of incubation, the cell culture medium was collected and an equal volume of sodium acetate buffer (200 mM, pH=4.5) with 0.1% L-ascorbic acid (Sigma, St. Louis, MO, USA) was added to stop the enzymatic

reactions. The sample of cell culture medium was frozen at -20 °C until the time for analysis E1 and its six metabolites.

The samples of cell culture medium (1.0 mL) were added to a deuterium-labeled internal standard, estrone-2,4,16,16-d4 (E1-d4) and then loaded onto a 96-well C18 solid-phase extraction plate (Discovery DSC-18 APE-96 plate, 100 mg/well, Supelco, Bellefonte, PA, USA). The C18 SPE wells were eluted with 100% methanol (2.0 mL) to recover the analytes. The resulting methanol solutions were dried under nitrogen and re-suspended in 50 μL for each well of HPLC loading buffer. An aliquot (20 μL) of sample solution from each well was injected to HPLC/MS/MS system for the quantification of analytes. HPLC/MS/MS experiments were performed using two Perkin-Elmer (Foster City, CA, USA) Series 200 micro pumps for gradient solvent delivery with the API 3000 mass spectrometer. Sample solutions were separated using a 15 cm x 4.6 mm i.d., particle size 5 μm, BDS Hypersil C18 column (Thermo Waltham, MA, USA) with 40-80% gradients of acetonitrile in aqueous at a flow rate of 600 μL min^{-1}.

Results and Discussion

The levels of E1 and its metabolites in the cell culture medium treated with E1 and various amounts of 2,3,7,8-TCDD for 24 hrs was demonstrated in Figure 2. The concentrations of E1 and its metabolites in the cell culture medium vary with the amounts of 2,3,7,8-TCDD added. E1 levels decreased when the cell culture medium was treated with 2,3,7,8-TCDD (Figure 2(a)). It is likely that E1 was converted into its metabolites, 2-OH E1, 4-OH E1, 16α-OH E1, 2-OMe E1, 4-OMe E1, and 2-OH-3-OMe E1, by cytochrome P450 and catechol O-methyltransferase (COMT) enzymes, as shown in Figure 1.

E1 was converted into its hydroxyl metabolites, 2-OH E1, 4-OH E1, 16α-OH E1, and the levels of E1 and its metabolites in the cell culture medium vary with the amounts of 2,3,7,8-TCDD added. Figure 2(b) shows that the levels of 2-OH E1 and 4-OH E1 in 2,3,7,8-TCDD-treated cell culture medium were higher than those in untreated cell culture medium and increased with the amount of 2,3,7,8-TCDD added. The levels of 2-OH E1 and 4-OH E1 in cell culture medium treated with 10 nM 2,3,7,8-TCDD were 4.6 (22/4.7) and 1.9 (5.0/2.7) times higher than those in untreated medium, respectively. The levels of 16α-OH E1 in 2,3,7,8-TCDD-treated cell culture medium were lower than that in untreated cell culture medium and decreased with the amount of 2,3,7,8-TCDD added (Figure 2(a)). The toxicant 2,3,7,8-TCDD is the most potent known CYP1A1 inducer[8]. These observations, i.e. levels of 2-OH E1 and 4-OH E1 increased with the amount of 2,3,7,8-TCDD added and 16α-OH E1 decreased with that, are consistent with previous reports[8,9]. The CYP1A1 exhibits relatively different catalytic activities for E1 hydroxylation at C2, C4, and C16[9]. It has the highest activity for E1 hydroxylation at C2, and the second highest for E1 hydroxylation at C4. For E1 hydroxylation at C16, the CYP1A1 has a low activity and these activities correlate with the present observations.

As shown in Figure 1, 2-OH E1 and 4-OH E1 were converted into metabolites, 2-OMe E1 and 4-OMe E1, by COMT, respectively, and the levels of the metabolites in the cell culture medium vary with the amounts of 2,3,7,8-TCDD added. Figure 2(c) shows that the levels of 2-OMe E1 and 4-OMe E1 in 2,3,7,8-TCDD-treated cell culture medium were higher than that in untreated cell culture medium and increased with the amount of 2,3,7,8-TCDD added. The levels of 2-OMe E1 and 4-OMe E1 in cell culture medium treated with 10 nM 2,3,7,8-TCDD were 7.7 (130/17) and 36 (16/0.45) times higher than those of untreated medium, respectively. There are at least two possible explanations for these observations. One possibility to be considered is that the increased levels of 2-OH E1 and 4-OH E1 may lead to a rapid subsequent metabolism, 2-OH-E2 to 2-MeO-E2, and 4-OH-E2 to 4-MeO-E2. Another possibility to be considered is that 2,3,7,8-TCDD treated largely increased the capability of COMT to metabolize 2-OH-E2 to 2-MeO-E2, and 4-OH-E2 to 4-MeO-E2. The association between the activity of the COMT enzyme and the amount of 2,3,7,8-TCDD added require further investigation. The level of 2-OH-3-OMe E1 was below the detection limit in all samples. All target analytes were below their detection limits in blank cell culture medium (untreated with E1 and/or 2,3,7,8-TCDD) or DMSO solution indicating no background interference of the target analytes.

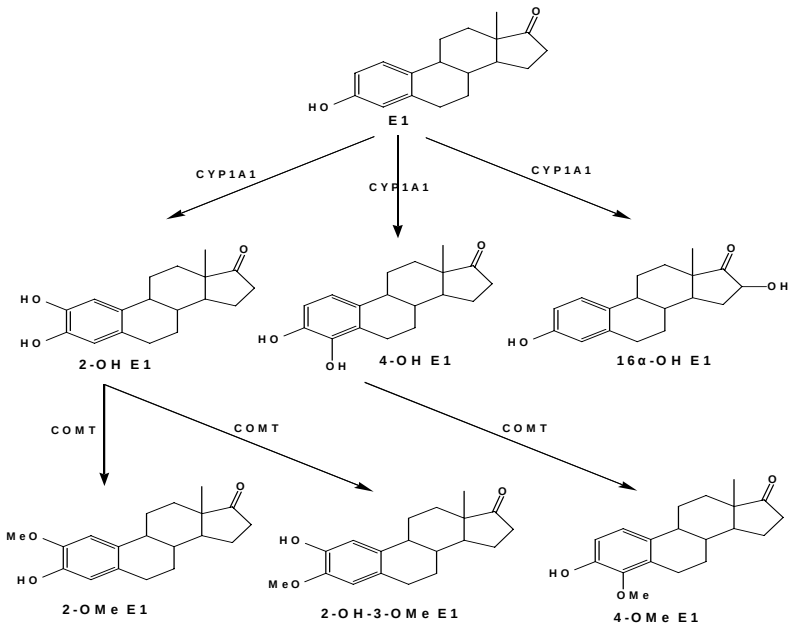
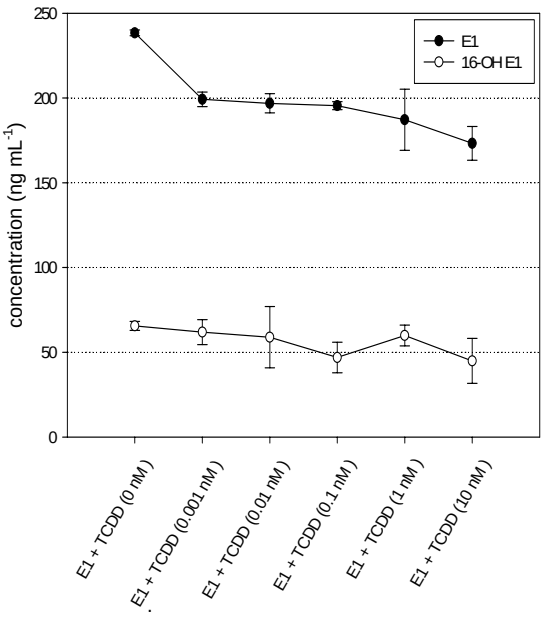
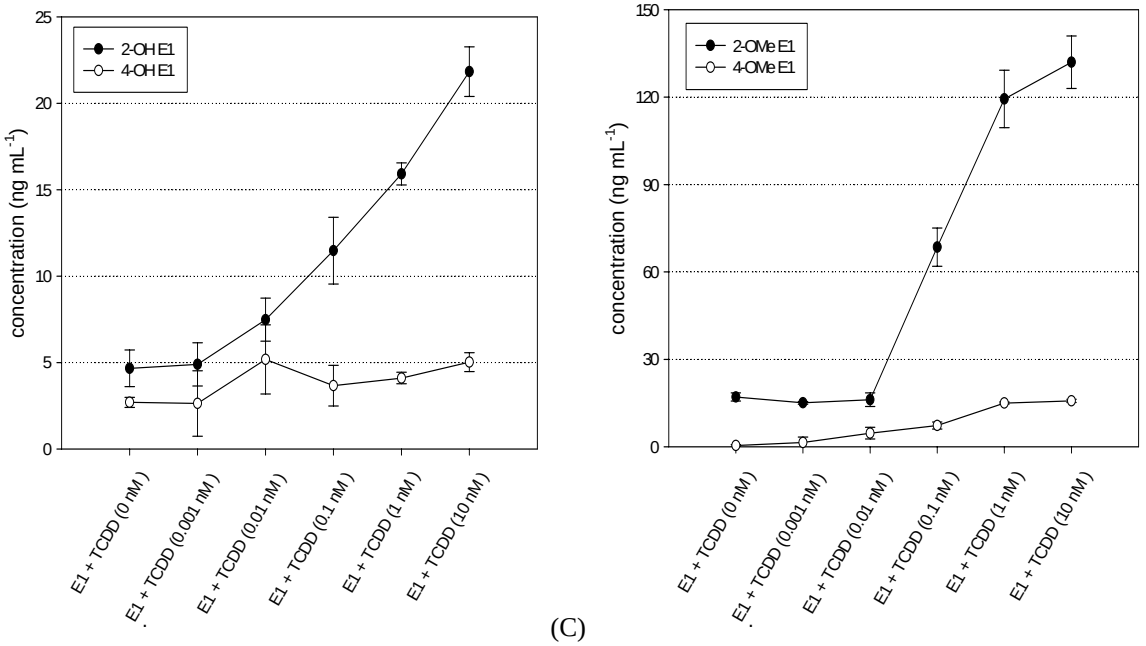


Figure 1. Formation of estrone metabolites. E1: estrone, 2-OH E1: 2-hydroxyestrone, 4-OH E1: 4-hydroxyestrone, 16α-OH E1: 16α-hydroxyestrone, 2-OMe E1: 2-methoxyestrone, 4-OMe E1: 4-methoxyestrone, 2-OH-3-OMe E1: 2-hydroxyestrone-3-methyl, CYP 1A1: cytochrome P450 1A1 family, and COMT: catechol O-methyltransferase.



(A)



(B) (C)

Figure 2. The levels of E1 and its metabolites in Human Hep G2 cells added by different amounts of 2,3,7,8-TCDD. (A) E1 and 16α-OH E1, (B) 2-OH E1 and 4-OH E1, (C) 4-OMe E1 and 2-OH-3-OMe E1

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