Novel Estrogenic Microsomal Metabolites from Phthalate Esters

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Many researchers have found evidence of the estrogenic effects of phthalate esters (PEs), but the disruption mechanism is not fully elucidated. Once PEs are hydroxylated at the ring 4-position, the resulting compounds exhibit unequivocal binding affinity for human estrogen receptors. In this study, we first succeeded to demonstrate the generation of potent estrogenic metabolite from PEs. We used rat liver microsomes and dimethyl phthalate (DMP) as a representative for the metabolism of PEs. Among the metabolites detected by HPLC, one of them was consistent with an authentic compound, 4-hydroxylated DMP (DMP-4OH) on their HPLC profiles. Together with the UV (λmax = 257 nm) and MS (*m*/*z* 210) data, the metabolite was concluded to be DMP-4OH. We propose that this ring-hydroxylated metabolite could be a key active species in the endocrine disrupting processes of PEs.

Key words —— phthalate ester, estrogenic activity, metabolic activation, ring-hydroxylation, microsome, endocrine disrupting chemical

INTRODUCTION

Phthalate esters (PEs) are considered endocrine disrupting chemicals, which have been manufactured in large quantities and used as plasticizers mainly for polyvinyl chloride (PVC). The most abundant PEs are di(2-ethylhexyl) phthalate (DEHP), and over 2 million tons are produced each year worldwide.[1] Since PEs are not covalently bound to the polymers, they leach from the matrix. Therefore, PEs are widely distributed in the atmosphere[2] and hydrosphere,[3,4] and thus many people are ubiquitously exposed to the PEs.[1,5,6] In medical care, disposable devices such as PVC bags and tubes plasticized with DEHP are used, and patients are directly exposed to DEHP.[7,8]

Some environmental chemicals express their estrogenic or anti-estrogenic activities through binding to estrogen receptors (ERs). These activities are mainly due to the structural similarity between these chemicals and original ligand, 17β-estradiol.[9] The crystal structure analysis of 17β-estradiol-bound ER revealed that the phenolic hydroxyl group of 17β-estradiol steroid A-ring is crucial for effective ER-binding.[10,11] PEs are potentially estrogenic, but the results on the potency obtained by many investigators are still inconsistent with each other. The inconsistency could be due to the extremely-weak ER-binding affinity of PEs.[12,13] However, PEs acquired unequivocal binding affinities for ERs when they were hydroxylated at the 4-position on the benzene ring.[14,15] Aromatic compounds are metabolically hydroxylated at the benzene ring by the first phase reaction. Therefore, PEs ingested or inhaled would disrupt the endocrine system in humans and animals *via* metabolic hydroxylation on the benzene ring. In this study, we have first succeeded to demonstrate that PEs are actually transformed into 4-hydroxylated PEs (PEs-4OH) using *in vitro* microsomal metabolic systems.

MATERIALS AND METHODS

Chemicals —— Dimethyl phthalate (DMP) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and β-nicotinamide adenine dinucleotide phosphate (β-NADP$^+$) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). 3-Methylcholanthrene and 3-hydroxyphthalic anhydride were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 4-Hydroxyphthalic acid was from Acros Organics Co., Ltd. (Geel, Belgium). Dimethyl

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4-hydroxyphthalate (DMP-4OH) and dimethyl 3-hydroxyphthalate (DMP-3OH) were synthesized according to the method reported by Carter *et al.*[16] Other chemicals used were of the highest quality commercially available.

Preparation of Rat Liver Microsomes —— Male Sprague-Dawley rats (5 weeks of age) were purchased from Clea Japan Inc. (Tokyo, Japan). The rats were given intraperitoneal administration of 3-methylcholanthrene (30 mg/kg body weight) daily for 3 days before use. After 24 hr of the final dose, the livers of rats were excised from exsanguinated rats and immediately perfused with 1.15% KCl. The microsomal pellets were suspended in 0.1 M sodium phosphate buffer (pH 7.4) to make 1 ml equivalent to 1 g of liver and stored at –70°C until use.

Incubation of DMP with the Microsomes —— The reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.4), DMP (5 mM), an NADPH-generating system (1 mM β-$NADP^+$, 10 mM glucose 6-phosphate, 1 unit/ml glucose 6-phosphate dehydrogenase, and 10 mM $MgCl_2$), and 2 mg microsomal protein in a final volume of 2 ml. The mixture was incubated at 37°C for 30 min, and the reaction was stopped by the addition of HCl. The metabolites were extracted with 2 ml of ethyl acetate two times. The organic layer was dried over anhydrous Na_2SO_4, and then concentrated below 30°C *in vacuo*.

HPLC Analysis —— The extracts from the microsomal reaction mixture were dissolved in methanol and applied to an HPLC system (LC-VP, Shimadzu, Kyoto, Japan) equipped with a diode array detector (SPD-M10A, Shimadzu) and a Develosil packed column (4.6 mm i.d. × 250 mm, Nomura Chemical Co., Ltd., Aichi, Japan) and eluted with 30% methanol/water containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min at 40°C.

GC/MS Analysis —— The samples were injected into a gas chromatograph (HP 6890 GC System Plus, Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a mass spectrometer (JMS-700 MStation, JEOL, Tokyo, Japan) using electron impact ionization at 70 eV. Helium was used as a carrier gas at a flow rate of 1 ml/min. The temperature of the injector, interface and ion source was 250°C. The temperature program for the DB-1 column (0.25 mm i.d. × 30 m, film thickness 0.25 μm, J&W Scientific, Folsom, CA, U.S.A.) was as follows: 70°C (2 min isothermal), 70–280°C (10°C/min) and 280°C (2 min isothermal).

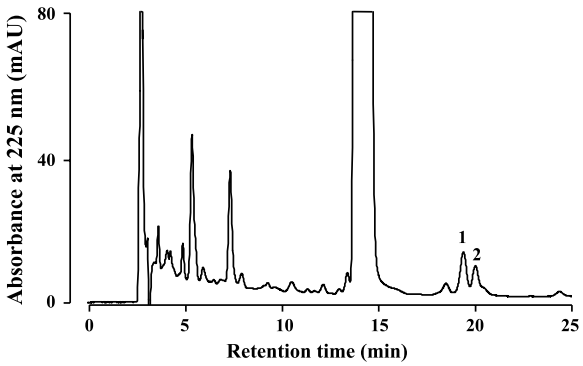

Fig. 1. HPLC Chromatogram of DMP Metabolites by Rat Liver Microsomes

RESULTS

HPLC chromatogram of DMP metabolites generated by rat liver microsomes is shown in Fig. 1. All peaks except the three peaks discussed below were also observed in the control that DMP was added into the microsomal reaction mixture after incubation. A major metabolite of DMP, monomethyl phthalate, was eluted in 14.2 min. Retention time of peak 1 (19.5 min) was consistent with that of a synthesized authentic DMP-4OH. UV spectrum of this peak (λmax = 257 nm) was almost identical to that of DMP-4OH with a matching factor of 99% (Fig. 2A). In the GC/MS analysis, the molecular ion mass of this peak was *m/z* 210, which was 16 larger than that of DMP (Fig. 3). The fragment ion mass (*m/z* 179) also preserved the difference. This indicates that the metabolite was formed by mono-oxygenation of DMP on the benzene ring. Retention time of peak 2 (20.1 min) was consistent with that of DMP-3OH which was another isomer of ring-hydroxylated DMP. Maximum UV absorption of peak 2 appeared at 300 nm, which was different from that of peak 1 (Fig. 2B). The molecular ion mass was *m/z* 210 and fragment ion masses were *m/z* 178, 148 and 120. This fragmentation pattern is characteristic of DMP-3OH. Conclusively, peaks 1 and 2 were attributed to DMP-4OH and DMP-3OH, respectively.

DISCUSSION

Metabolism of PEs undergoes hydrolysis of one ester followed by oxidation of the remaining ester chain and/or conjugation.[17,18] In addition, this study

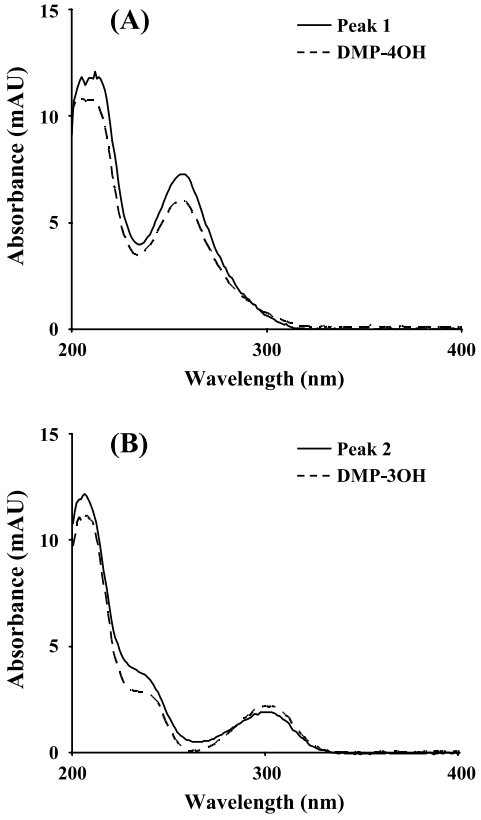

Fig. 2. UV Spectra of DMP Metabolites and Authentic Standards, DMP-4OH and DMP-3OH

revealed the existence of a new metabolic pathway, an aromatic ring-hydroxylation. The significance of this finding is not limited to the elucidation of the metabolic fate of administered PEs. One of the resulting metabolites, PEs-4OH, is clearly positive in assays for estrogenic activity measurement.[14,15] Estrogenic potencies of PEs themselves are not sufficient to explain the adverse effects caused by exposure to PEs. Therefore, the present finding suggests a possibility that PEs exhibit their estrogenic activity through metabolic conversion to PEs-4OH.

Not much attention has been paid to the adverse effects of PEs compared to other environmental chemicals, although several PEs possess estrogenic properties. This is because PEs are rapidly excreted from the body through hydrolysis to their respective monoesters which are further oxidized and conjugated to increase solubility. Moreover, estrogenic potencies of PEs and the primary metabolite, monoesters, are almost negligible; for instance, the ER-binding affinity of di-*n*-butyl phthalate which is the strongest ER-binder among PEs is approximately 30000 times less than that of 17β-estradiol.[19] The detailed mechanism of endocrine disruption caused by PEs is not fully elucidated, although several possibilities such as the antiandrogenic effect[20] and sup-

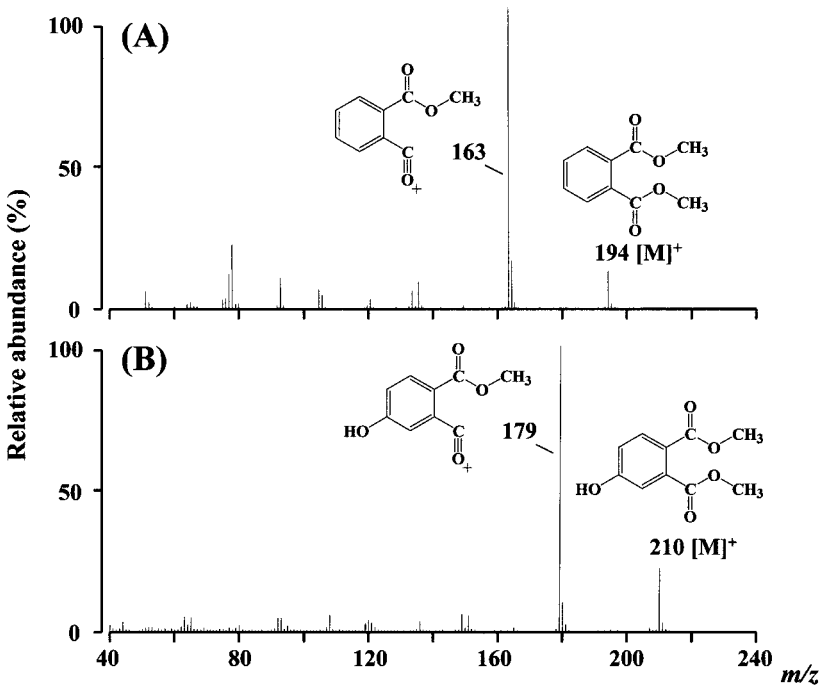

Fig. 3. Mass Spectra of DMP and Peak 1
(A), DMP; (B), peak 1.

pression of 17β-estradiol production[21] have been proposed. This uncertainty suggests that PEs express their estrogenic activities through metabolic transformations. Once PEs are hydroxylated at the ring 4-position, they acquire unequivocal binding affinity for ERs due to the similarity to 17β-estradiol and turned to be capable of induction of various effects caused by the ER-mediated manner. Therefore, these ring-hydroxylated derivatives could be a key active species in the endocrine disrupting processes of PEs.

Recent research has revealed that ER regulates the expression of a diverse set of genes which were concerned not only with reproductive functions,[22] suggesting that exposure to PEs would exert broader adverse effects than previously thought. The present results give an insight into the mechanism of endocrine disruption by PEs and deserve further investigation to evaluate the risk of exposure to PEs.

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