— **Review** —

Potential Risks of Phthalate Esters: Acquisition of Endocrine-disrupting Activity during Environmental and Metabolic Processing

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Phthalate esters (PEs) are plasticizers used in many plastic products, food packaging materials, and medical bags. The widespread use of PEs has led to ubiquitous environmental contamination and human-exposure. Several epidemiological studies have indicated that exposure to PEs, especially during the prenatal period, induces adverse effects, including developmental and behavioral abnormalities, suggesting that PEs are endocrine-disrupters. Although the endocrine-disrupting effect of PEs is thought to be estrogen receptor (ER)-mediated, the ER-binding affinity of PEs is quite low, indicating that other mechanisms should be considered. In this review, we demonstrate that alkyl chain length and hydroxylation of PEs have a significant impact on binding to ERs and peroxisome proliferator-activated receptors. In addition, we discuss recent results from our laboratory that suggest additional risks may arise from environmental and metabolic processing of PEs through microbial transformation, microsomal metabolism, and sunlight exposure.

Key words —— plasticizer, light exposure, microsomal metabolism, microbial biotransformation, estrogen receptor, peroxisome proliferator-activated receptor

INTRODUCTION

Although phthalate esters (PEs) are suspected endocrine-disrupting chemicals, they are manufactured in large quantities and widely used as plasticizers for plastic products such as food packaging materials and medical bags. One of the most abundant PEs is di(2-ethylhexyl) phthalate (DEHP), over two million tons of which is produced each year worldwide.[1)] PEs are not covalently bound to plastic polymers, and as a result they leach from the matrix and become widely distributed in the atmosphere[2)] and hydrosphere.[3, 4)] For example, indoor concentrations of DEHP range from 0.04 to 0.23 $\mu g/m^3$, and inside a car may range from 1 to 34 $\mu g/m^3$.[5)] During medical treatments that involve infusion, such as hemodialysis and peritoneal dialysis therapies or parenteral nutritional therapy, patients may be exposed to several milligrams of DEHP from bags and tubes.[6, 7)] Most importantly, several studies have indicated that infants and toddlers, who are highly susceptible to PEs, are extensively exposed to these compounds.[8–10)] Considerable evidence suggests that many people are routinely exposed to PEs on a daily basis.[1, 11, 12)]

Many epidemiological studies have suggested that PE-exposure, especially during the prenatal period, induces a number of adverse effects in humans, such as developmental and behavioral abnormalities.[13–15)] It has also been reported that PE-exposure is correlated with inflammation, oxidative stress, and DNA damage.[16–19)] The broad effects of PEs have led to speculation that they target hormone receptors, such as estrogen receptors (ERs) and peroxisome proliferator-activated receptors (PPARs). In order to evaluate the relevance of these receptors with respect to PE-induced adverse effects, it is necessary to understand the environmental and metabolic fate of PEs, especially because of the potential risks associated with environmental and metabolic processing.

Given their widespread use, PEs eventually enter soil or water environments (rivers, lakes, or the sea), where they may be transformed into ac-

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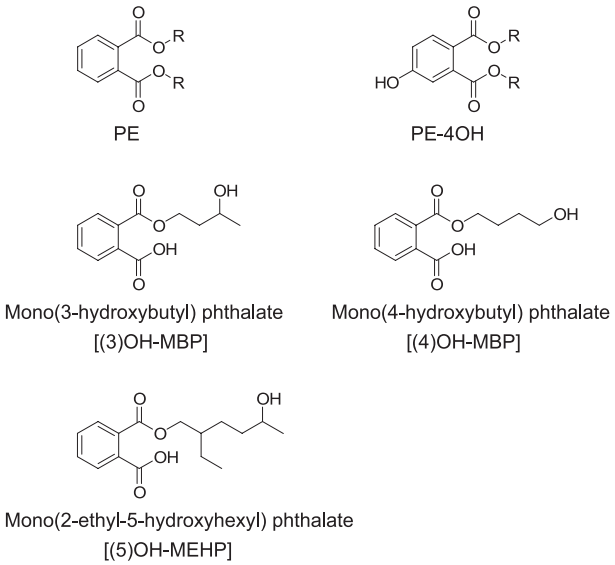

Fig. 1. Basic Structures of PE, 4-Hydroxylated PE, and Alkyl Chain Hydroxylated Derivatives

tive derivatives by microbial degradation or exposure to sunlight. PEs are also found in the air and dust, where they may be transformed into active derivatives by sunlight exposure and then inhaled (air) or ingested (dust) and further metabolized. This review describes the dynamics of the ER- and PPAR-mediated effects of PEs, based on structure-activity relationships. In addition, novel PE transformation pathways based on model experiments simulating microbial biotransformation, microsomal metabolism, and sunlight exposure are also described.

ER-MEDIATED EFFECTS OF HYDROXYLATED PHTHALATE ESTERS

Numerous *in vitro* studies employing purified human ERs, genetically engineered yeast strains, and mammalian cell lines have indicated that PEs such as benzyl butyl phthalate (BBP), di-*n*-butyl phthalate (DBP), and di-*n*-propyl phthalate (DPP) are estrogenic.[20−28] However, because the estrogenic activity of these PEs is extremely weak compared to natural estrogen 17β-estradiol (E2), the implications of these *in vitro* studies are controversial.[29] Moreover, since all PEs tested are non-estrogenic in female rats,[30, 31] there is considerable uncertainty regarding interpretation of data from animal experiments and human epidemiological studies.

Although PEs are primarily metabolically processed through hydrolysis, they may also be hydroxylated by cytochrome P450, as are many xenobiotics. We synthesized several ring- and alkyl chain-hydroxylated derivatives (Fig. 1) and assayed their effect on ER-mediated activity *in vitro*. In an ER-binding assay using human ERα and ERβ, ring 4-hydroxylated PEs (PEs-4OH) bound preferentially to ERα, while 3-hydroxylated PEs did not.[32] The binding affinity of PEs-4OH increased with alkyl chain length as follows: methyl $\ll$ ethyl $<$ hexyl $<$ propyl $<$ butyl $<$ pentyl $<$ heptyl $<$ octyl for ERα, and methyl $\ll$ ethyl $<$ propyl $<$ hexyl $<$ pentyl $<$ butyl $<$ octyl $<$ heptyl for ERβ. The contribution of each PE-4OH alkyl chain was observed more effectively when the alkyl chains were substituted at the 1-position.[33] In addition, alkyl chain branching increased the ER-binding affinity of the PEs-4OH, while alkyl chain hydroxylation had no effect, suggesting that ring-hydroxylation as well as the structure of the alkyl chain (length and branching) are the key features influencing the ER-binding of PEs.

Transcription factors such as coactivators must be recruited to specific genome sequences (estrogen-responsive elements) for the expression of ER target genes. PEs-4OH with shorter alkyl chains (from methyl to butyl) recruit the coactivator transcriptional intermediary factor 2 (TIF2), whereas PEs-4OH with longer alkyl chains (from pentyl to octyl), such as DEHP-4OH, have no transcription factor recruiting activity.[33] Ligand-dependent relocation of helix12, which composes the ligand-binding domain of ERs, determines whether a ligand is an agonist or antagonist.[34, 35] Longer alkyl chain PEs-4OH disrupt agonistic relocation of helix12 to the coactivator docking site due to steric hindrance, thereby precluding coactivator-recruitment in the same manner as the ER antagonists raloxifene and ICI 182,780.[33−35]

PPAR-MEDIATED EFFECTS OF HYDROXYLATED PHTHALATE ESTERS

Some PEs (*e.g.*, DEHP) have been classified as peroxisome proliferators because they induce peroxisomal and hepatocellular proliferation in rodents. These adverse effects are associated with the activation of PPARs by hydrolyzed PE metabolites rather than by the parent diester. A primary hydrolyzed metabolite, mono(2-ethylhexyl)phthalate (MEHP), has been identified as the effecter responsible for DEHP-induced abnormalities in ro-

dents. Experiments using hepatic cell lines demonstrated that MEHP activates PPARα-reporter gene expression.[36, 37] Scintillation proximity binding assay experiments revealed that MEHP interacts with PPARα.[38]

PPARγ is a target in diabetes treatment. PPARγ agonists such as thiazolidinedione rosiglitazone improve insulin resistance in patients with type 2 diabetes by modifying adipocyte differentiation.[39] Several groups using reporter gene and receptor binding assays demonstrated that MEHP activates PPARγ.[36–38]

In addition to being metabolized through hydrolysis, PEs can be produced ring- or alkyl chain-hydroxylated metabolites.[40–43] Using several oxidized metabolites of DBP and DEHP (Fig. 1), we examined the coactivator-recruiting activity of human PPARα/γ (hPPARα/γ).[44] DBP, monobutyl phthalate (MBP), mono(4-hydroxybutyl) phthalate [(4)OH-MBP], and MBP-4OH did not induce any coactivator-recruiting (agonistic) activity in hPPARα, although mono(3-hydroxybutyl) phthalate [(3)OH-MBP] demonstrated a weak ability to induce coactivator recruitment. DEHP and its metabolites such as MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate [(5)OH-MEHP], MEHP-4OH, and DEHP-4OH had little to no ability to induce coactivator-recruiting activity. Based on the results of a reporter gene assay, Bility *et al*. (2004) reported weak agonistic activity of PEs with respect to hPPARα.[37]

To examine antagonistic effects against hPPARα, we tested inhibition of coactivator-recruiting activity induced by the PPARα agonist GW7647.[44] Among DBP, DEHP, and their metabolites, only DBP-4OH demonstrated a dose-dependent antagonistic effect. With respect to hPPARγ, DBP induced weak coactivator-recruiting (agonistic) activity, whereas DBP metabolites did not. The DEHP metabolite MEHP acted as a PPARγ agonist. These results indicate that hydroxylation of PEs tends to decrease hPPARγ-mediated effects. To examine antagonistic effects against hPPARγ, we examined inhibition of coactivator-recruiting activity induced by the PPARγ agonist rosiglitazone.[44] Coactivator recruiting was inhibited by DBP, DBP-4OH, MBP, and MEHP, whereas (4)OH-MBP, DEHP, and its oxidized metabolites had no antagonistic effect.

An epidemiological study revealed that increased inflammation and oxidative stress are correlated with phthalate monoesters, but not with their oxidized metabolites.[19] Inflammation induced by PE-exposure may be due to activation of PPARα or inhibition of PPARγ.[18, 45] Because hPPARα-mediated transactivation are negligible,[37, 44] the increased inflammation and oxidative stress resulting from exposure to PEs might be associated with inhibition of hPPARγ. Such a scenario would explain the inverse correlation between oxidative stress and exposure to oxidized metabolites.[19]

MICROBIAL BIOTRANSFORMATION

Biotransformation of PEs involves the sequential hydrolysis of each ester-alkyl chain, which is accompanied by the formation of phthalic acid and various monoesters.[46–48] However, it is unclear whether additional transformation products contribute to the toxicity of PEs. Since the biological effects of PEs fluctuate depending on the length and degree of branching of the alkyl chain,[32, 33, 37, 44] it is important to determine the environmental fate of these alkyl chains in order to perform adequate human and environmental risk assessments.

Our group isolated PE-degrading microbes from river water (Tempaku River, Nagoya, Japan) and determined their ability to biodegrade diethyl phthalate (DEP), DPP, DBP, and DEHP.[3] We found that biotransformation of DBP resulted in the formation of dimethyl phthalate (DMP), monomethyl phthalate (MMP), MBP (a typical DBP-degradation product), and an unknown diester. This suggests the existence of a novel PE biotransformation pathway, and it is possible that the above-mentioned transformation products contribute to the adverse effects associated with PEs. The unknown diester was also produced in the biotransformation of DPP, although the HPLC retention time of the unknown diester was different from that of DBP. Based on LC/MS and ^{1}H-NMR analyses, the unknown diester biotransformation product of DPP was identified as methyl propyl phthalate. Asymmetrical diesters have also been reported by two other groups. Cartwright and co-workers (2000) reported that soil bacteria degrade DEP to ethyl methyl phthalate in the presence of CH_3OH.[49] Without performing additional experiments, they concluded that ethyl methyl phthalate was produced by the demethylation of the ethyl group. Kim *et al*. (2002) reported the formation of butyl methyl phthalate during the biodegradation of benzyl butyl phthalate by fungal cutinase and yeast esterase in the presence of CH_3OH.[50] To

determine the source of the methyl group in methyl propyl phthalate, we carried out a stable-isotope tracer experiment using CD_3OH as a PE-solvent.[48] An HPLC peak with a retention time consistent with methyl propyl phthalate was detected in the experiment; however, the molecular weight of the compound as determined by LC/MS was 3 mass units larger than that of methyl propyl phthalate. Furthermore, no ^{1}H-NMR signal that could be assigned as the methyl ester group of methyl propyl phthalate was observed. The use of dimethyl sulfoxide as DPP-solvent in the biotransformation experiment precluded observation of methyl propyl phthalate in the HPLC chromatogram. These data indicated that the methyl ester group is derived from CH_3OH. Asymmetrical diesters can be produced via esterification of phthalate monoesters or enzymatic/non-enzymatic transesterification of phthalate diesters. However, biotransformation of phthalate monoester in the presence of CH_3OH and the absence of microbes in the cultivation medium did not result in the formation of asymmetrical diesters. Asymmetrical diesters are thus thought to be formed via enzymatic transesterification of phthalate diester and alcohol.

MICROSOMAL METABOLISM

As the biological effects of PEs vary depending upon structural modifications, the identification and detailed analysis of PE metabolites is important. PEs are primarily hydrolyzed by esterases to produce monoesters. Novel metabolites resulting from microsomal metabolism of DMP have also been identified.[43] Based upon UV spectra and GC/MS analyses, these microsomal metabolites were identified as ring 3- or 4-hydroxylated DMP. The potent ER-mediated activity of PEs-4OH suggests that PEs acquire ER-mediated activity via metabolic processing. Inhibition of PE-4OH production in rat liver microsomes by the nonspecific P450 inhibitor SKF525A suggests this is probably a result of oxygenation by cytochrome P450 enzymes (Okamoto *et al.*, unpublished data).

EXPOSURE TO SUNLIGHT

PEs contaminating the air and water environments may be exposed to sunlight, resulting in the formation of photodegradation products. While several groups have investigated the feasibility of removing PEs from water using light-irradiation and photo-catalytic materials,[51, 52] until recently very little was known about the potential toxicity of photodegradation products. To the best of our knowledge, our group was the first to describe the toxicity of PE photodegradation products formed by sunlight exposure.[53] We found that several photoproducts are formed after DPP is exposed to sunlight or UV irradiation. One product showed unequivocal estrogenic activity, whereas DPP itself did not. Using UV and LC/MS analyses, we determined that the estrogenic photoproduct was DPP-4OH, which is generated by exposure of DPP to UV irradiation at 290 nm. Production of DPP-4OH is accompanied by H_2O_2 generation in a UV dose-dependent manner (*i.e.*, longer exposure to UV leads to increased H_2O_2 generation). Both DPP and UV irradiation are required for the generation of H_2O_2. Addition of H_2O_2 to the DPP solution increased DPP-4OH production under UV irradiation. DPP-4OH production was also observed in the reaction of DPP with the Fenton reagent, generating hydroxyl radicals without UV irradiation. Based on these results, we propose that as a result of PE-mediated photosensitization, H_2O_2 is generated from O_2 and H^+ and then decomposes to hydroxyl radicals, thus oxidizing the PE benzene ring. The production of DPP-4OH as a result of sunlight exposure represents a significant problem since PEs-4OH are remarkably active estrogenic products and would be expected to be involved in ER-mediated endocrine disruption.

CONCLUSION

In this review, we described how benzene ring and alkyl chain modifications (hydroxylation, chain length, and branching) affect the endocrine disrupting activity of PEs. Ring 4-hydroxylation plays a key role in the ER-mediated biological effects of PEs, while variation in alkyl chain structure is also important. Hydrolyzed metabolites (monoesters) with long alkyl chains have potent hPPARγ-mediated biological effects, which are diminished upon oxidation of the PE. The data discussed here help explain the epidemiological observations that indicate exposure to oxidized metabolites is inversely associated with inflammatory and oxidative stress induced by exposure to the parent

PEs. The data also suggest that the environmental and biological fates of PEs are more complex than previously thought. Therefore, PE risk assessments should be more comprehensive, taking into consideration potential risks that may arise as a result of environmental and metabolic processing of PEs.

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