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Effects of phthalate ester derivatives including oxidized metabolites on coactivator recruiting by PPARα and PPARγ

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ABSTRACT

Phthalate esters (PEs), a group of environmental chemicals, affect biological systems via endocrine and lipid metabolism modulations. These effects are believed to be mediated in part by peroxisome proliferator-activated receptors (PPARs). Evaluations of PE activities as ligands toward PPARs have been investigated in many studies on their primary metabolites, monoesters. However, the activities of various other metabolites, including oxidized derivatives, remain to be determined. Here, we have evaluated the PPAR ligand activities of these PE derivatives by in vitro coactivator recruiting assay. Mono(2-ethyl-5-hydroxyhexyl)phthalate, the most abundant metabolite of di-(2-ethylhexyl)phthalate (DEHP), was less active than mono(2-ethylhexyl)phthalate (MEHP) as a PPAR ligand. Other derivatives oxidized at the alkyl group and benzene ring of DEHP, MEHP, dibutyl phthalate and its monoester were also investigated and some affected PPAR activities. Unexpectedly, MEHP as well as its further oxidized metabolite did not show clear activity for PPARα, although MEHP is believed to interact with PPARα. This might imply indirect PPAR-mediated mechanisms that lead to observed biological effects such as peroxisome proliferation.

1. Introduction

Phthalate esters (PEs) represent a group of plasticizers to provide flexibility to plastic products and are considerable environmental contaminants. PEs leach from such products due to the lack of chemical binding to the matrix, and thus are commonly detected in the environment (Hashizume et al., 2002). Use of PEs for medical treatments such as blood transfusion is of particular concern because relatively high concentrations of contaminating PEs are occasionally detected (Haishima et al., 2004; Inoue et al., 2005). Recent population studies have detected PEs in human urine and milk (Blount et al., 2000; Calafat et al., 2004).

Some PEs such as di(2-ethylhexyl)phthalate (DEHP) are recognized as peroxisome proliferators based on the observations in rodents. For instance, DEHP induces the enzymes involved in fatty acid catabolism such as CYP4A and acyl-CoA oxidase, which are involved in ω- and β-oxidations, respectively (Barber et al., 1987; Bell and Elcombe, 1991). The induction of these enzymes is mediated by peroxisome proliferator-activated receptor α (PPARα) (Ward et al., 1998). The target genes have a PPAR-binding sequence, called PPRE (peroxisome proliferator responsive element), in the 5′-region (Muerhoff et al., 1992; Tugwood et al., 1992). A primary hydrolyzed metabolite, mono(2-ethylhexyl)phthalate (MEHP), has been identified as the responsible effecter for DEHP-dependent enzyme induction. MEHP activated reporter gene expression in the experiments using hepatic cell lines (Hurst and Waxman, 2003), and interacted with PPARα using scintillation proximity binding assay (Lapinskas et al., 2005). On the other hand, PPARγ is recognized as a target in diabetes treatment; PPARγ agonists, such as thiazolidinedione rosiglitazone, improve insulin resistance in type 2 diabetes by modifying adipocyte differentiation (Simonson and Kendall, 2006). MEHP was also found to activate PPARγ using the reporter and binding assays (Hurst and Waxman, 2003; Lapinskas et al., 2005).

However, it is uncertain whether MEHP is the only responsible metabolite for the toxicity of DEHP. PEs such as dibutyl phthalate (DBP) and DEHP are also known to be metabolized into derivatives other than monoesters, including their glucuronized products, alkyl-truncated monoesters and oxidized intermediates, using rodents (Tanaka et al., 1978; Albro et al., 1982; Foster et al., 1982). In humans, urinary oxidized metabolites have been recognized as an indicator of exposure to PEs (Barr et al., 2003; Kato et al., 2004). In a recent report by Koch et al. 23.3% of the applied dose of DEHP was detected as mono(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP) in the urine at 24 h after oral intake, in addition to mono(2-ethyl-5-carboxypentyl)phthalate (18.5%), mono(2-ethyl-5-oxohexyl)phthalate (15.0%), and MEHP (5.9%) (Koch et al.,

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2005a). These levels were similar to the results obtained by intravenous administration (Koch et al., 2005b), and in good agreement with the observations of former studies (Schmid and Schlatter, 1985; Dirven et al., 1993). The effects of these oxidized metabolites have not been evaluated for their effects on PPARs, despite their abundance during PE metabolism. In the present study, we investigated the effects of oxidized PE metabolites on coactivator recruiting by PPARα and PPARγ. Based on the results, we evaluate the risk of these metabolites, and discuss the possibility of indirect PPARα-mediated mechanisms underlying the toxicological effects induced by PEs and their metabolites.

2. Materials and methods

2.1. Chemicals

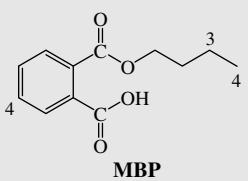
MEHP, DEHP, DBP, butanol, 1,3-butanediol, 1,4-butanediol, ethyl hexanol, imidazole and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-Hydroxyphtalic anhydride was from Acros Organics (Geel, Belgium). Silica gel (BW-300) was from Fuji Silysia Chemical (Aichi, Japan). Trifluoroacetic acid was from Merck (Darmstadt, Germany). MBP was from Tokyo Kasei Kogyo (Tokyo, Japan). 5OH-MEHP was from Cambridge Isotope Laboratories (Andover, MA). *t*-Butyldimethylchlorosilane was from Shin-Etsu Chemical (Tokyo, Japan), and *t*-butyldiphenylchlorosilane was from Alfa Aesar (Ward Hill, MA). Di(2-ethylhexyl)4-hydroxyphthalate (DEHP-4OH) and dibutyl 4-hydroxyphthalate (DBP-4OH) were laboratory stock prepared previously (Toda et al., 2004). Abbreviations for PE derivatives are summarized in Table 1.

2.2. Preparation of PE derivatives

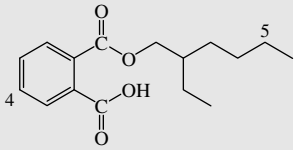
Monobutyl 4-hydroxyphthalate (MBP-4OH) and mono(2-ethylhexyl)4-hydroxyphthalate (MEHP-4OH) were prepared from 4-hydroxyphthalic anhydride and the corresponding silylated alcohols, as described previously (Carter et al., 1977; Toda et al., 2004). Mono(3-hydroxybutyl)phthalate (3OH-MBP) and mono(4-hydroxybutyl)phthalate (4OH-MBP) were synthesized from 4-hydroxyphthalic anhydride and silylated 1,3-butandiol and 1,4-butandiol, respectively, according to the method for DEHP derivatives (Gilsing et al., 2002). 4-Butandiol was silylated with *t*-butyldimethylchlorosilane, and 1,3-butandiol was silylated with *t*-butyldimethylchlorosilane and *t*-butyldiphenylchlorosilane to reduce undesirable products. Reaction products were purified using silica gel and TLC (silica gel 60 F254, Merck) with chloroform–methanol–water (10:1:0.1, v/v/v), followed by HPLC (Jasco, Tokyo, Japan) with a Develosil column ODS-HG-5 (20 mm i.d. × 250 mm, Nomura Chemical, Aichi, Japan) with a mobile phase of 80% (v/v) methanol–water containing 0.1% trifluoroacetic acid at a flow rate of 9 ml/min. Concentrations of 3OH-MBP and 4OH-MBP were determined by comparing peak areas (225 nm) with MBP on analytical HPLC (LC-VP, Shimadzu, Kyoto, Japan). LC/MS consisted of an HPLC system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA) and an LCQ-DECA XP Plus ion-trap mass spectrometer (Thermo Electron, San Jose, CA). Positive ions were scanned under the following conditions: ion spray voltage, 5 kV; capillary voltage, 3.14 V; capillary temperature, 275 °C. Compounds were diluted with DMSO to obtain solutions of desired concentration for assays, although 3OH-MBP and 5OH-MEHP required prior concentration in an evaporator. These phthalate ester derivatives are listed in Table 1.

Table 1
Phthalate ester derivatives used in this study

Compound	Abbreviation
Monobutyl phthalate	MBP
Monobutyl 4-hydroxyphthalate	MBP-4OH
Mono(3-hydroxybutyl) phthalate	3OH-MBP
Mono(4-hydroxybutyl) phthalate	4OH-MBP
Dibutyl phthalate	DBP
Dibutyl 4-hydroxyphthalate	DBP-4OH
Mono(2-ethylhexyl) phthalate	MEHP
Mono(2-ethylhexyl) 4-hydroxyphthalate	MEHP-4OH
Mono(2-ethyl-5-hydroxyhexyl) phthalate	5OH-MEHP
Di(2-ethylhexyl) phthalate	DEHP
Di(2-ethylhexyl) 4-hydroxyphthalate	DEHP-4OH



MBP



MEHP

2.3. Coactivator-recruiting assay

Ligand-dependent coactivator-recruiting to PPARs was measured using the NuLigand kit (Microsystems, Kyoto, Japan). This assay is based on the CoA–BAP system in which activity was determined by bacterial alkaline phosphatase (BAP) fused to the nuclear receptor interaction domain of a coactivator interacting with the GST-hPPAR ligand-binding domain immobilized on a plate (Kanayama et al., 2003, 2005). PPAR solution diluted with 0.1 M carbonate buffer was applied to plate wells and incubated at 2–8 °C overnight. After washing the wells, coactivator–BAP solution and test compound were added and incubated on ice for 1 h. Substrate solution was added to the washed wells and plates were left to stand at 30 °C until the solution mixture was colored (0.5–3 h). After addition of 0.5 M NaOH, A_{405} was measured. Activity is presented as% of positive controls (GW7647 for PPARα; rosiglitazone for PPARγ). In order to evaluate antagonistic activity, procedures were performed in the presence of 10^{-7} M of a positive control. Inhibitory effects are presented as% of the activity obtained for a positive control.

3. Results

3.1. Effects of PEs on coactivator recruiting by PPARα

DBP and its metabolites, MBP and 4OH-MBP were not potent PPARα agonists in coactivator recruiting experiment (Fig. 1A). Although 3OH-MBP showed increased activity at 10^{-5} M, the activity decreased in the higher concentration. DEHP showed weak activity increasing with its concentrations, while its metabolites, MEHP and 5OH-MEHP, did not (Fig. 1B). Antagonistic effects were also investigated in the presence of a control agonist GW7647 (Fig. 2A and B). The effects in this assay system were confirmed using known antagonist GW9662. DBP and MBP did not show inhibitory effect, whereas hydroxylated derivatives such as DBP-4OH showed dose-dependent inhibition (Fig. 2A). Neither DEHP nor its metabolites showed antagonistic activity for PPARα, although 5OH-MEHP could not be investigated at lower concentrations than 10^{-6} M (Fig. 2B).

3.2. Effects of PEs on coactivator recruiting by PPARγ

DBP exhibited weak coactivator recruiting activity in PPARγ only at the highest concentration, and its derivatives were inactive in all concentration range tested (Fig. 3A). MEHP showed a dose-dependent increase of the agonistic activity for PPARγ (Fig. 3B).