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Effect of Di-(2-ethylhexyl)-phthalate on Sphingolipid Metabolic Enzymes in Rat Liver

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Di-(2-ethylhexyl)-phthalate (DEHP), the most widely utilized industrial plastizer and a ubiquitous environmental contaminant, can act on peroxisome proliferators-activated nuclear hormone receptor family (PPAR) isoforms. To understand the contribution of sphingolipid metabolism to DEHP-induced hepatotoxicity, effect of DEHP exposure on activities of sphingolipid metabolic enzymes in rat liver was investigated. DEHP (250, 500 or 750 mg/kg) was administered to the rats through oral gavage daily for 28 days. The activities of acidic and alkaline ceramidases were slightly increased in 250 mg/kg DEHP-administered rat livers and significantly elevated in 500 mg/kg DEHP-administered ones, although the level of 750 mg/kg DEHP-administered ones was not increased. Neutral ceramidase, acidic and neutral sphingomyelinases, sphingomyeline synthase and ceramide syhthase were not changed at all by DEHP exposure. Therefore, acidic and alkaline ceramidases might play important roles in DEHP-induced hepatotoxicity.

***Key words*:** Di-(2-ethylhexyl)-phthalate, Sphingosine, Sphingomyelinase, Ceramidase, Liver, Sphingomyelin synthase, Ceramide synthase

INTRODUCTION

Di-(2-ethylhexyl)-phthalate (DEHP), the most widely utilized industrial plastizer and a ubiquitous environmental contaminant, have been the focus in recent years and are believed to have a greater relevance to humans (Kavlock *et al.*, 2002). DEHP belongs to a diverse class of peroxisome proliferator chemicals that can act on peroxisome proliferators-activated nuclear hormone receptor family (PPAR) isoforms (Dzhekova-Stojkova *et al.*, 2001). It has been suggested that DEHP might affect placental handling and fetal essential fatty acid/lipid supply via PPAR trans-activation, as indicated by the up-regulation of certain PPAR isoforms and fatty acid transporters in the placenta to the fetus upon exposure to DEHP (Xu *et al.*, 2005). Also in a rat HRP-1 trophoblast model, exposure of DEHP and its metabolites increased levels of ten lipid classes (Xu *et al.*, 2006). Furthermore, it was shown that *in utero* exposure to DEHP alters the lipid metabolome in the fetal brain (Xu *et al.*, 2007).

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Sphingolipids are family of lipid second messenger molecules that regulate various aspects of the cell functions (Cuvillier, 2002; Spiegel *et al.*, 1996). Sphingolipid metabolites play important roles in cell proliferation and apoptosis (Cuvillier, 2002; Spiegel *et al.*, 1996). Ceramide and sphingosine in particular, mediate cellular responses to cytokines, serum deprivation, γ-irradiation, or other stress conditions. By means of exquisite homeostatic mechanisms, the liver maintains the distinctive capacity to regulate its tissue mass by inductive cell division and apoptosis. Hepatocellular injury activates signal transduction pathways that mediate cell repair, proliferation, or even cell death (Chalfant *et al.*, 2002; Chen *et al.*, 1995; Chun *et al.*, 2003; Cock *et al.*, 1998). Interestingly, bioactive sphingolipids are increasingly appreciated as important participants in liver responses to a variety of perturbations. A number of extracellular stimuli are known to induce ceramide levels in cells, including tumor necrosis factor-α (TNF-α), Fas ligand, lipopolysaccharide (LPS or endotoxin), and chemotherapeutic agents (Hannun, 1996). Furthermore, many of these inducers of ceramide are also known to be involved in mediating liver injury. Peroxisome proliferators including perfluorodecanoic acid (PFDA) and DEHP caused a decrease in sphingomyelin probably via TNF-α in rat liver (Adinehzadeh and Reo, 1998).

DEHP is rapidly metabolized in liver to mono-(2-ethyl-

hexyl)-phthalate. However, its effect on liver has not been reported, although its chronic exposure enlarged the mass of liver (Kim *et al.*, 2010; Ryu *et al.*, 2007). Therefore, in this study we tested whether DEHP exposure affect activities of sphingolipid metabolic enzymes such as neutral Mg^{2+}-dependent sphingomyelinase (N-SMase) and acid sphingomyelinase (A-SMase), ceramidases (acid, neutral and alkaline), sphingomyelin synthase, and ceramide synthase.

MATERIALS AND METHODS

Materials. [N-methyl-^{14}C]-sphingomyelin was purchased from Sigma (St. Louis, MO, USA), and [N-palmitoyl-1-^{14}C]-sphingosine from Moravek Biochemicals (Brea, CA, USA). D-*erythro*, [3-^{3}H]-sphingosine and E^{3} Enhancer were procured from PerkinElmer LAS, INC (Boston MA, USA). D-*erythro*-sphingosine (synthetic) was purchased from Avanti Polar Lipids Inc. (Alabaster, USA). Silica gel HPTLC (60 F_{254}, 20 × 20) was obtained from Merck (Darmstadt, Germany) and Kodak Medical X-ray film from Eastman Kodak Company (NY, USA). All other materials were purchased from Sigma-Aldrich Korea (St. Louis, MO, USA).

Animal and treatment. Male Sprague-Dawley rats (3 weeks of age, weighing approximately 60~70 g) were obtained from the Charles River Laboratories (Japan) and housed under a controlled temperature (22 ± 2°C) and lighting (12 hr light/dark cycle). The animals were given access to an animal diet (PMI, Brentwood, MO) and tap water *ad libitum*. DEHP (250, 500 or 750 mg/kg) was administered to the rats through oral gavage daily for 28 days. The control rats were administered corn oil in the same manner.

Preparation of tissue homogenates. Rat liver homogenates were prepared as described by others (Igarashi and Hakomori, 1989) with some modifications. Tissues including kidney, liver and brain were removed from Sprague-Dawley rats as described above and washed in cold phosphate-buffered saline (PBS) separately. The tissues were placed in 10 m*l* of cold 20 mM Tris-HCl (pH 7.5) and 2 mM EDTA solution, homogenized by Tekmar homogenizer (OH, USA) at 4°C. The homogenate was centrifuged at 500 *g* for 10 min to remove unbroken cell debris, and the supernatant was used as an enzyme source for sphingomyelinase, ceramidase, sphingomyelin synthase and ceramide synthase.

The activity of sphingomyelinase. The activity of N- and A-SMases was determined as reported by Liu and Hannun (Liu and Hannun, 2000) with slight modifications. Briefly, the tissue homogenates were centrifuged at 1000 *g* for 10 min and the supernatant (~5 mg of protein) was used for further analysis. The activity of both SMases was measured using radiolabeled substrate, [N-methyl-^{14}C]-sphingomyelin. For N-SMase, the reaction mixture contained 100 nmol of sphingomyelin (1154 dpm/nmol) in 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1% Triton X-100 and 5 mM dithiothreitol in a final volume of 0.2 m*l*.

In the case of A-SMase, the assay mixture contained 100 nmol of sphingomyelin (1154 dpm/nmol) in 100 mM sodium acetate (pH 5.0), 0.1% Triton X-100 and 0.1 mM EDTA. After incubation at 37°C for 1 h the reaction was stopped by adding 1.5 m*l* of chloroform: methanol (2 : 1), followed by 0.2 m*l* of water. A portion of the aqueous phase was transferred to scintillation vials and counted in a liquid scintillation counter for the radioactivity of the reaction product, ^{14}C-choline phosphate.

The activity of ceramidases. The activity of Al-CDase, N-CDase and Ac-CDase ceramidase was determined by the method of Nikolove-Karakashian and Merrill (Nikolova-Karakashian and Merrill, 2000) with slight modifications. The activity of the enzymes was measured using radiolabeled substrate [N-palmitoyl-1-^{14}C]-sphingosine. The tissue homogenates were centrifuged at 1000 *g* for 10 min and the supernatants (~5 mg of protein) were used for further analysis. The reaction was initiated by addition of supernatant to the tubes containing 20 μ*l* of substrate mixture (50 nmol of ceramide - 2353 dpm/nmol, 2.5 mg Triton X-100, 1 mg Tween 20, 0.4 mg sodium cholate) and 130 μ*l* of a reaction buffer. The reaction buffer contained 125 mM sucrose, 0.01 mM EDTA and 125 mM sodium acetate (pH 4.5) or 100 mM Tris-HCl (pH 7.2) or 125 mM HEPES (pH 8.0) for Ac-CDase, N-CDase and Al-CDase activity assay, respectively. After incubation at 37°C for 1 h the reaction was stopped by adding 2 m*l* of basic Doyle's solution (isopropanol : heptane : 1 N NaOH, 40 : 10 : 1, v/v/v), 1.8 m*l* of heptane and 1.6 m*l* of water. Samples were then centrifuged and the upper phase was discarded. The lower phase was washed twice with 1.6 m*l* heptane and then 1 m*l* of 1 N H_2SO_4 and 2.4 m*l* of heptane were added. After centrifugation, 1 m*l* aliquots from the upper phase were transferred to scintillation vials and analyzed for the radioactivity of the reaction product, ^{14}C-palmitate.

The activity of sphingomyelin synthase. The activity of sphingomyelin synthase was determined as reported by Luberto and Hannun (Luberto and Hannun, 1998) with slight modifications. In brief, the reaction contained 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 0.5 mM EDTA (Futerman *et al.*, 1990) and homogenate containing ~5 mg of protein. The reaction was initiated by addition of [N-palmitoyl-1-^{14}C]-sphingosine (20 nmol) as an equimolar complex with fatty acid free bovine serum albumin (complex specific activity: ~9×10^3 cpm/nmol) and allowed to proceed for 60 min. The reaction was stopped by addition of 3 m*l* of chloroform : methanol (1 : 2); the mixture was vortexed and kept on ice. Lipids were extracted as indicated by the Bligh

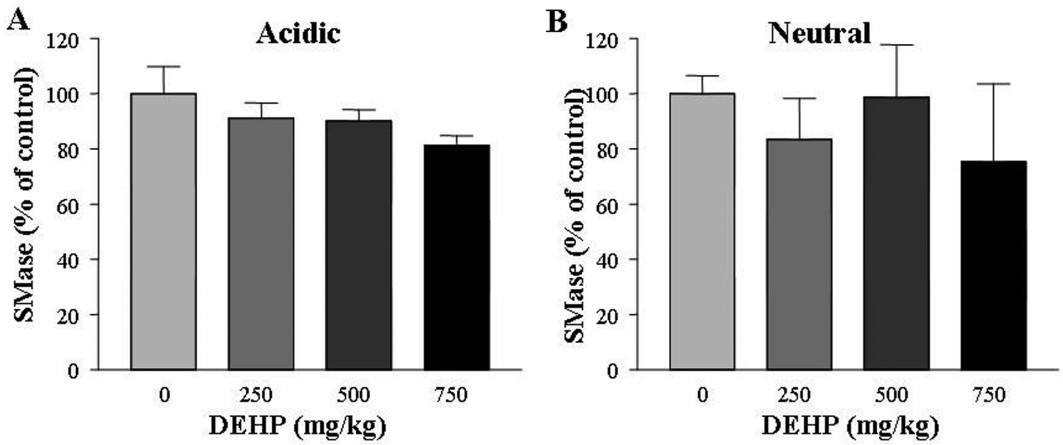

Fig. 1. SMase activity in rat liver. Acidic sphingomyelinasee (A) and neutral sphingomyelinase (B) activities were measured from DEHP-exposed rat liver for 28 days. Rat tissue homogenates were incubated with [N-methyl-^{14}C]–sphingomyelin. Lipids were extracted and radioactivity of reaction product ^{14}C-choline phosphate counted in a liquid scintillation counter.

and Dyer method (Bligh and Dyer, 1959) and resolved by TLC in chloroform : methanol : 15 mM anhydrous $CaCl_2$ (60 : 35 : 8). The [N-palmitoyl-1-^{14}C]-SM produced was detected by autoradiography, scraped from the plates and quantitated by liquid scintillation counting. Values for blanks were subtracted from total values of [N-palmitoyl-1-^{14}C]-SM to yield the amount of [N-palmitoyl-1-^{14}C]-SM produced.

The activity of ceramide synthase. The assay mixture for sphingosine N-acyltransferase contained 1 μM [^{3}H]sphingosine, 25 mM potassium phosphate buffer (pH 7.4), 0.5 mM dithiothreitol, 200 μM palmitoyl-CoA, and approximately 0.2 mg of microsomal protein in a total volume of 0.1 m*l* as reported previously (Morell and Radin, 1970). The reaction was initiated by adding palmitoyl-CoA, and after incubation at 37°C for 15 min, the products were extracted, resolved by TLC, and quantitated as described above. Background counts were subtracted using data from identical assays that omitted palmitoyl-CoA.

Statistical analysis. The results are expressed as mean ± SE of three determinations. Statistical significance of differences was determined by student-*t* test. Significance was accepted when $p < 0.05$.

RESULTS

Effect of DEHP exposure on sphingomyelinase activity in liver. There are two kinds of sphingomyelinases, that is acidic and neutral sphingomyelinases. We measured sphingomyelinase activity of liver homogenates from DEHP (0, 250, 500, 750 mg/kg)-treated rats for 28 days both in acidic condition and in neutral condition. In rat liver homogenates acidic sphingomyelinase activity was two times higher than neutral sphingomyelinase. However, as shown in Fig. 1, exposure of DEHP did not influence the activities (Fig. 1).

Effect of DEHP on ceramidase activity in liver. There are three kinds of ceramidases, that is acidic, neutral, and alkaline ceramidases. We measured ceramidase activity of liver homogenates from DEHP (0, 250, 500, 750 mg/kg)-treated rats for 28 days in acidic, neutral, and alkaline conditions. As shown in Fig. 2A, in rat liver homogenates acidic ceramidase activity was increased in liver homogenates from 250 and 500 mg/kg DEHP-treated rats. However, the activity in 750 mg/kg DEHP-treated rat liver homogenate was not changed. In rat liver homogenates neutral ceramidase activity was slightly increased in liver homogenates from 250, 500, and 750 mg/kg DEHP-treated rats (Fig. 2B). As shown in Fig. 2C, in rat liver homogenates alkaline ceramidase activity was also increased in liver homogenates from 250 and 500 mg/kg DEHP-treated rats. However, the activity in 750 mg/kg DEHP-treated rat liver homogenate was not changed.

Effect of DEHP on sphingomyelin synthase and ceramide synthase activity in liver. A second enzyme that may contribute to the accumulation of ceramide is sphingomyeline synthase. Sphingomyeline synthase (phosphatidylcholine : ceramide phosphorylcholinetransferase) catalyzes the transfer of the phosphocholine head group of phosphatidylcholine to ceramide, thus forming sphingomyeline (Lightle *et al.*, 2000). To investigate whether DEHP affected the activity of this enzyme, we measured the sphingomyeline synthase activity in livers from DEHP-treated rats using [N-palmitoyl-1-^{14}C]-sphingosine as the substrate. We observed that sphingomyeline synthase activity was not changed as like sphingomyelinase activity (Fig. 3).

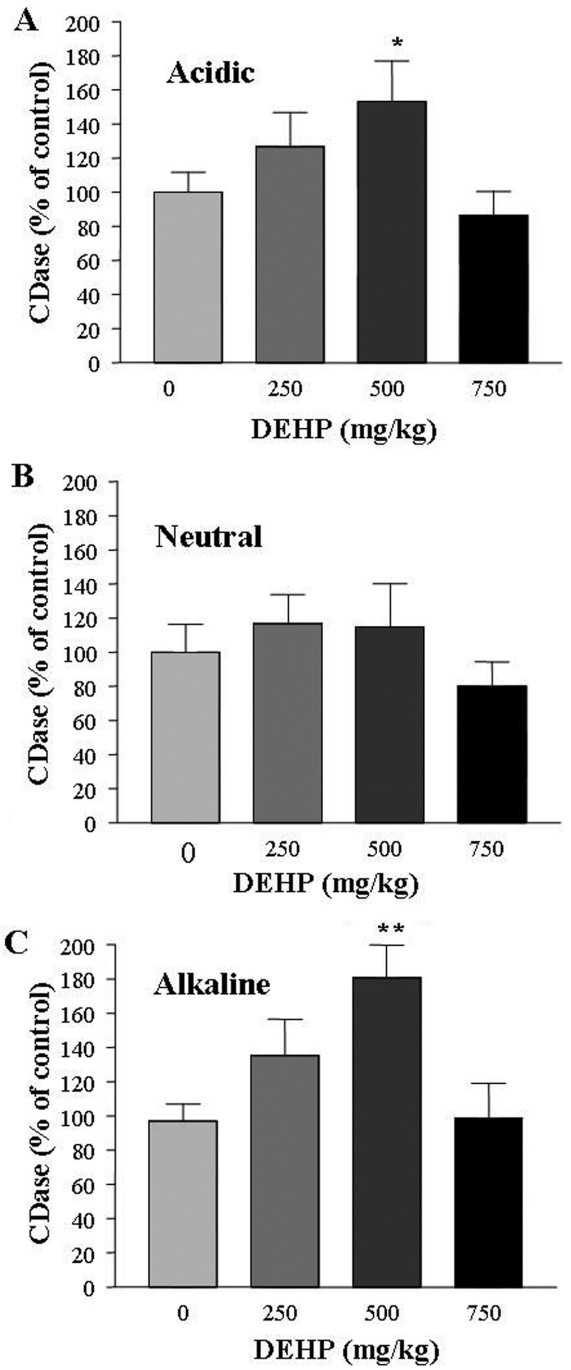

Fig. 2. Ceramidase activity in rat liver. Acidic (A), neutral (B) and alkaline (C) ceramidase activities were measured from DEHP-exposed rat liver for 28 days. Rat liver homogenates were incubated with [N-palmitoyl-1-^{14}C]–sphingosine. Lipids were extracted and radioactivity of reaction product ^{14}C-palmitate was counted in a liquid scintillation counter. Statistical significance: ** $P < 0.01$, * $P < 0.05$ vs control.

Next, we measured ceramide synthase activity in the liver homogenates. We found that the enzyme activity was not much changed by the treatment of DEHP in rat liver (Fig. 4).

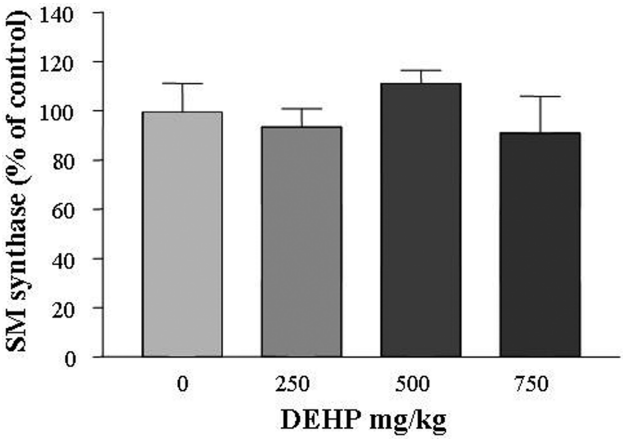

Fig. 3. Sphingomyeline synthase activity in rat liver. Sphingomyeline synthase activity was measured from DEHP-exposed rat liver for 28 days. Rat liver homogenates were incubated with [N-palmitoyl-1-^{14}C]–sphingosine. Lipids were extracted and [N-palmitoyl-1-^{14}C]-SM isolated on TLC by autoradiography and quantitated using a liquid scintillation counter.

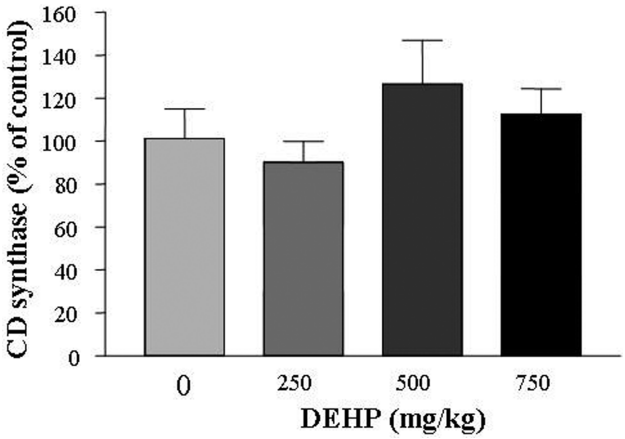

Fig. 4. Ceramide synthase activity in rat liver. Ceramide synthase activity was measured from DEHP-exposed rat liver for 28 days. Rat liver homogenates were incubated with [^{3}H]–sphingosine. Lipids were extracted and [^{3}H]–ceramide isolated on TLC by autoradiography and quantified using a liquid scintillation counter.

DISCUSSION

Di-(2-ethylhexyl)-phthalate (DEHP) is the most widely utilized industrial plastizer and a ubiquitous environmental contaminant. By studying sphingolipid metabolic enzyme activities in DEHP-exposed rat livers, we found DEHP could increase activities of acidic and alkaline ceramidases but not neutral ceramidase. And none of sphingomyelinases, sphingomyeline synthase and ceramide synthase were changed by DEHP exposure. Ceramidases can regulate the cellular ceramide level by hydrolyzing ceramide to sphingosine and free fatty acids. This enzyme not only regulates

the levels of ceramide, but also that of sphingosine.

Ceramidases are key enzymes regulating cellular levels of apoptosis-related sphingolipids, ceramide and sphingosine. Activation of acidic ceramidase has been linked to resistance of ceramide-mediated death pathways. For instance, overexpression of acidic ceramidase was shown to protect cells from TNF-α-induced apoptosis (Strelow *et al.*, 2000). We observed that ceramidase (Ac-CDase and Al-CDase) activities increased specifically in DEHP-exposed rat liver tissue. Increase in the ceramidase activity in the liver might suggest decrease of ceramide levels and increase of sphingosine generation by the exposure in the tissues. Because lipid profiling study of DEHP showed a decrease in sphingomyelin content immediately after the exposure in rat liver (Adinehzadeh and Reo, 1998), further investigation on the levels of ceramide and sphingosine would be useful to interpret the present data.

We only studied livers after 28-day exposure with DEHP. Therefore, we do not know the activity changes during the treatment especially at the dose of 750 mg/kg DEHP. Although the level of 750 mg/kg DEHP-administered ones was not increased at 28-day exposure, the activities of ceramidases might be increased during the treatment and declined back to the basal level by the higher toxicity of the dose. Further experiments on the time course of sphingolipid metabolic enzyme activities during hepatotoxicity would be needed.

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