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Diethyl phthalate exposure is associated with embryonic toxicity, fatty liver changes, and hypolipidemia via impairment of lipoprotein functions

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ABSTRACT

Diethyl phthalates (DEPs) are notorious for their high potential toxicity in endocrinological and reproduction systems in humans and animals. In this study, we investigated the toxic effects of DEP on human lipoproteins, macrophages, and zebrafish embryos. Treatment of human high-density lipoprotein (HDL) with DEP caused oxidation, aggregation, and degradation of lipoproteins. DEP treatment promoted foam cell formation via accelerated phagocytosis of LDL by macrophages as well as exacerbated cellular senescence in human dermal fibroblasts. Injection of DEP (final 5 μM and 10 μM) into zebrafish embryos caused severe embryo death and slower developmental speed. Exposure of zebrafish embryos to water containing DEP (final 11 and 22 ppm) caused early embryonic death along with the increased oxidized products and impairment of skeletal development. Adult zebrafish exposed to water containing DEP (final 11 and 22 ppm) for 4 weeks showed severe loss of body weight under both normal diet (ND) and high cholesterol diet (HCD) conditions. ND and HCD groups showed 59% and 49% reduction of plasma total cholesterol (TC), respectively. Serum levels of hepatic inflammation enzymes along with fatty liver changes were significantly elevated by DEP exposure. In conclusion, DEP showed strong pro-atherogenic and pro-senescence effects via severe lipoprotein modification in human cells. DEP caused impairment of embryonic development and severe loss of body weight, hypolipidemia, and fatty liver changes in zebrafish.

1. Introduction

Phthalates are used for the industrial production of various items such as plastics and medical devices and have received attention due to their high potential toxicity in humans and animals (Jobling et al., 1995; Rozati et al., 2002; Parkerton and Konkel, 2000; Staples et al., 1997). Among phthalates, DEP is widely used as a plasticizer to increase the flexibility of polymer in many products (Kang et al., 2010). Because DEP can be existed in numerous cosmetic formulations and such as hair sprays, perfumes, and shampoos (Kamrin and Mayor, 1991), DEP can be easily absorbed via inhalation and dermal exposure (Duty et al., 2005; Silva et al., 2004), whereupon they may act as toxicants and interfere with normal function of the endocrine system (Heudorf et al., 2007; Soto et al., 1995; Uren-Webster et al., 2010) or as endocrine disruptors and interfere with the fish immune system (Milla et al., 2011). Among the many types of phthalates, DEP is used widely in personal care products as a denaturant and fixative (Hauser and Calafat, 2005). DEP shows a wide range of toxicities in gastrointestinal and cardiovascular systems, inducing significant changes in the activities of certain liver and muscle enzymes (Ghorpade et al., 2002) or showing reproductive effects in infants, toddlers, and pregnant women or women of a childbearing age (Casas et al., 2011; Wormuth et al., 2006). In addition, DEP shows reproductive toxicity in males (Martino-Andrade and Chahoud, 2010; Matsumoto et al., 2008). Although their mechanism of toxicity is unknown, DEP has been shown to have deleterious effects on human health, specifically exacerbating metabolic diseases such as diabetes and cardiovascular disease (CVD).

HDL-cholesterol is inversely related with incidence of coronary heart disease. HDL is a protein and lipid complex in plasma that exerts potent antioxidant, anti-inflammatory, and anti-atherosclerotic activities (Barter et al., 2004). HDL also plays a protective role against the development of CVD, whereas low-density lipoprotein (LDL) oxidation is correlated with increased risk of CVD (Gordon et al., 1977; Linsel-Nitschke and Tall, 2005; Barter et al., 2004). ApoA-I, the major protein of HDL, is responsible for mediating several beneficial effects in HDL. Many researchers, including our group, have reported that HDL quality is highly dependent on the structural and functional correlations of apoA-I during aging (Park et al., 2010; Park and Cho, 2011a). Modification of apoA-I is directly related with production of dysfunctional HDL, which has strong atherogenic and inflammatory effects that exacerbate cellular senescence (Park et al, 2014). Taken together, reports by our

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research group along with others strongly suggest that the functionality of HDL is highly affected by the serum composition, including environmental hormones. Modification of lipoproteins is a key step in the development of atherosclerosis (Rye and Barter, 2014; Hadfield et al., 2013).

Although serum exposure to DEP has been shown to have potential toxicity in humans and animals (Steinberg, 2002; Sonde et al., 2000), their molecular mechanism has not been clearly established. As DEPs that are ingested either orally or dermally are released into the bloodstream, interactions between DEP and lipoproteins should be investigated. Especially, functional and structural correlations of HDL can be modified by foreign molecules such as acrylamide (Kim et al., in press) and artificial sweeteners (Kim et al., 2011, 2015). Since there has been no report on interactions between DEP and lipoproteins, we investigated the effects of DEP on lipoprotein metabolism both in vitro and in vivo in human cells, adult zebrafish, and zebrafish embryos.

Zebrafish (*Danio rerio*) and zebrafish embryos have been used as models of hyperlipidemia (Kim et al., 2011) and inflammation (Park and Cho, 2011a). To investigate the physiologic effects of DEP in vertebrate animals, we used a zebrafish (*D. rerio*) model in which hypercholesterolemia is induced by a high cholesterol (HC) diet for 4 weeks, as reported previously by others and our research group (Stoletov et al., 2009; Kim et al., in press; Kim et al., 2011). Zebrafish have well-developed innate and acquired immune systems that are very similar to the mammalian immune system (Trede et al., 2001). As an additional advantage, zebrafish embryos develop externally and are optically transparent during development. Due to these characteristics, zebrafish are a useful and popular animal model for a variety of studies, including those related to inflammation (Novoa et al., 2009) and oxidative stress (Fang and Miller, 2012).

In the current study, we investigated the molecular mechanism by which DEP exacerbate fatty liver changes and impairment of embryonic development via lipoprotein modification in humans and zebrafish in order to better understand their promotion of diabetes, atherosclerosis, and infertility.

2. Materials and methods

2.1. Materials

Diethyl phthalate (Cat # 524972) was purchased from Sigma-Aldrich (St. Louis, MO). Palmitoyloleolyoylphosphatidylcholine (POPC, #850457c) and cholesterol was obtained from Avanti Polar Lipids (Alabaster, AL).

2.2. Purification of lipoproteins and apoA-I

Human plasma was isolated by low-speed centrifugation from healthy males who donated blood voluntarily and fasted for at least 16 h before bleeding. Very low-density lipoprotein (VLDL, $d < 1.019$ g/mL), low-density lipoprotein (LDL, $1.019 < d < 1.063$), high-density lipoprotein (HDL_2, $1.063 < d < 1.125$), and HDL_3 ($1.125 < d < 1.225$) were isolated via sequential ultracentrifugation, and densities were appropriately adjusted by the addition of NaCl and NaBr in accordance with standard protocols (Havel et al., 1955). Samples of each lipoprotein were centrifuged for 22 h at 10 °C and 100,000 *g* using a Himac CP-90α (Hitachi, Tokyo, Japan) at the Instrumental Analysis Center of Yeungnam University. After centrifugation, each lipoprotein was extensively dialyzed against Tris-buffered saline (TBS; 10 mM Tris–HCl, 5 mM EDTA, and 140 mM NaCl [pH 7.4]) for 24 h in order to remove NaBr.

For in vitro analysis, human apoA-I in a lipid-free state was purified from HDL by ultracentrifugation and column chromatography as described previously (Brewer et al., 1986). Protein concentration in a lipid-bound state was determined according to a modified Lowry protein assay for lipoproteins as described previously (Markwell et al., 1978) using bovine serum albumin as a standard.

2.3. Synthesis of rHDL containing fatty acids

Discoidal rHDL containing fatty acids was prepared by the sodium cholate dialysis method (Matz and Jonas, 1982; Cho, 2011) at an initial molar ratio of 95:5:1:x:150 for palmitoyloleoyl phosphatidylcholine (POPC):free cholesterol (FC):apoA-I:DEP:sodium cholate (x represents 1, 10, or 100). For example, rHDL(1:100) indicates a molar ratio of 95:5:1:100:150 for POPC:FC:apoA-I:DEP:sodium cholate. We synthesized rHDL(1:1), rHDL(1:10), and rHDL(1:100). The rHDL particles were used without further purification since they showed high homogeneity.

Their sizes were determined using 8–25% native polyacrylamide gradient gel electrophoresis (PAGGE, Pharmacia Phast system, GE healthcare, Uppsala, Sweden) and compared with those of standard globular proteins (Cat# 17-0445-01GE healthcare, Uppsala, Sweden). Relative migrations were compared via densitometric scanning analysis using a Gel Doc® XR (Bio-Rad, Hercules, CA, USA) with Quantity One software, version 4.5.2.

2.4. Modification of lipoproteins to DEP

Human HDL_3 (1.5 mg/mL) or lipid-free apoA-I (1.5 mg/mL) was incubated with DEP (0.05, 0.5, 5, 50 μM) for the designated time up to 72 h at 37 °C in the presence of 5% CO_2. After incubation, extent of modification was analyzed by fluorospectroscopy to detect yellowish fluorescence intensity at 370 nm (Excitation) and 440 nm (emission) as described previously (Kim et al., in press). Electrophoretic patterns of incubated HDL were characterized by 15% SDS-PAGE. Human LDL treated with DEP for 48 h was analyzed by 6% SDS-PAGE and agarose gel to compare electrophoretic patterns and electromobility as previously described (Noble, 1968).

2.5. LDL oxidation and acetylation

Low-density lipoprotein (LDL) was oxidized by treatment with $CuSO_4$ (final 10 μM) for 8 h at 37 °C LDL (oxLDL), followed by electrophoresis on 0.5% agarose gels to compare relative electromobility (Noble, 1968). Acetylation of LDL (acLDL) was performed using saturated sodium acetate and acetic anhydride according to a previously described method (Fraenkel-Conrat, 1957). After acetylation and subsequent dialysis, protein content of acLDL was determined and the particles filtered through a 0.22-mm filter (Millex; Millipore, Bedford, MA) prior to use.

2.6. LDL phagocytosis assay

THP-1 cells, a human monocytic cell line, were obtained from the American Type Culture Collection (ATCC, #TIB-202™, Manassas, VA, USA) and maintained in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum until needed. Cells that had undergone no more than 20 passages were incubated in 24-well plates at 37 °C in a humidified incubator (5% CO_2), which was water-jacketed. The monocyte was supplemented with medium containing phorbol 12-myristate 13-acetate (PMA, 150 nM) for 48 h in order to induce differentiation into macrophages. Differentiated and adherent macrophages were then rinsed with warm PBS and incubated with 400 μL of fresh RPMI-1640 medium containing 1% FBS, acLDL (50 μg of protein in PBS), and 50 μL of DEP-treated HDL_3 for 48 h at 37 °C in the humidified incubator. After incubation, cells were washed with PBS three times and then fixed in 4% paraformaldehyde for 10 min. Next, fixed cells were stained with oil-red O staining solution (0.67%) and then washed with distilled water. THP-1 macrophage-derived foam cells were then observed and photographed using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) at 600× magnification.