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Di-N-octylphthalate acts as a proliferative agent in murine cell hepatocytes by regulating the levels of *TGF-β* and pro-apoptotic proteins

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ABSTRACT

Di-n-octylphthalate (DNOP) is a phthalate used in the manufacturing of a wide variety of polyvinyl chloride-containing medical and consumer products. A study on chronic exposure to DNOP in rodents showed the development of pre-neoplastic hepatic lesions following exposure to a tumor initiator. The objective of this study was to identify the mechanisms by which DNOP leads to pre-neoplastic hepatic lesions. Mouse hepatocyte AML-12 and FL83B cells were treated with DNOP. The rate of cell proliferation was increased in treated cells in a concentration-dependent manner. DNOP increased the expression of transforming growth factor-β (tgf-β) in both cell lines, and primary culture mouse hepatocytes. The TGF-β receptor inhibitor LY2109761 impaired the effect of DNOP. The presence of pro-apoptotic proteins decreased in the presence of DNOP. Our observation indicates that DNOP, through an increase in the expression of tgf-β and a decrease in the levels of pro-apoptotic proteins, acts as a proliferative agent in normal mouse hepatocytes. We also studied the morphological and functional changes of the mouse liver upon a short-term treatment of DNOP. Mice exposed to DNOP displayed an epithelial-to-mesenchymal transition and cholestasis, which was reflected in an increase in hepatic bile acids and glutathione levels.

1. Introduction

The impact of plasticizers on hepatotoxicity is a major concern in public health due to their extensive use in industrial production (Rusyn and Corton, 2012). Phthalates such as di-n-octylphthalate (DNOP) are used as plasticizers, which are chemicals that make plastic flexible. DNOP is 20% of a phthalate mixture used in flooring, pool liners, and garden hoses (National Toxicology and P., 2003). The phthalate is also found in cosmetics, adhesives, and wire cables along with food and food packaging, as DNOP is approved by the Food and Drug Administration as an indirect food additive (National Toxicology and P., 2003; Silva et al., 2005).

Once in the body, phthalates follow a similar pattern of metabolism and have a half-life in the scope of hours in humans (Schecter et al., 2013). In the liver, the original compound is hydrolyzed into its monoester, mono-n-octylphthalate, which is oxidized into water soluble metabolites and eliminated with urine. High doses of DNOP can result in a longer time period needed for elimination of DNOP and its metabolites. Rats fed 300 mg/kg of DNOP still showed levels of DNOP metabolites in their urine after 4 days (Silva et al., 2005). While the presence of DNOP and its metabolites in the urine decreased 95% after one day, the metabolites were essentially eliminated in few days (Silva et al., 2005). DNOP residues were found in liver (4–5 ppm ~ detection limit) and adipose tissue (15–25 ppm) 13 weeks following dietary exposure to 5000 mg/kg of DNOP in rats (Poon et al., 1997). The cause of concern is not the high dose from a particular object, but the ubiquitous presence of the substance in everyday objects.

As previously mentioned, DNOP is in indirect food products, such as bottle cap liners and food conveyer belts, and DNOP's presence in food is not surprising since DNOP is not covalently bound to plastic, making it easier to leach out into surroundings (National Toxicology and P., 2003).

Consumers are not the only ones at risk of DNOP exposure. Manufacturing of products using DNOP exposes workers and contaminates industrial solid and water wastes, as well as air emissions (Silva et al., 2005). The estimated human exposure in the United States is 3–30 μg/kg/day, but exposure can be 143–286 μg/kg/day for workers in the manufacturing of DNOP products (Silva et al., 2005).

Although there is not much information on the levels of DNOP in humans in the United States, a Hong Kong study found that out of 153

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samples of collected human blood, over 95% contained DNOP with the mean level being 6.53 ng/ml ± 3.95 (Wan et al., 2013); this study suggests the presence of DNOP in humans, at least in this area, is common.

Though the product is ubiquitous, more information on the chemical is needed as DNOP is listed as a probable hepatotoxic chemical under the Federal Hazardous Substances Act (The Federal Hazardous Sub, 2010). Two separate studies showed that DNOP acted as a tumor promoter in rats. After 10 or 26 weeks of feeding a diet containing 10,000 ppm DNOP, a number of pre-neoplastic hepatic lesions initiated with diethylnitrosamine were seen in male Sprague-Dawley and Fischer rats (DeAngelo et al., 1986; Carter et al., 1992). Regarding the mechanism by which these lesions are developed, peroxisome proliferation is thought to be the non-genotoxic mechanism. Peroxisome proliferation has been demonstrated to be of importance in phthalate-induced hepatocellular tumorigenesis (David et al., 1999). Di(2-ethylhexyl) phthalate (DEHP), which is the straight chain isomer of DNOP causes peroxisome proliferation (Ito et al., 2007); however, DEHP has induced liver tumors in peroxisome proliferator-activated receptor (PPAR)α-null mice, suggesting that peroxisome proliferation by phthalates is not necessary for liver tumor development (Veras et al., 2009). In addition, DNOP causes limited peroxisome proliferation, which has been attributed to the rapid metabolism of DNOP into lower molecular weight metabolites (Poon et al., 1997; Mann et al., 1985; Hinton et al., 1986; Lake et al., 1984a, 1984b; Bility et al., 2004), indicating the existence of another mechanism.

Exploring the possibility of one or multiple mechanisms other than peroxisome proliferation is relevant to human health as PPARα activation leading to liver tumor development is seen in rodents but limited in humans (Veras et al., 2009).

The objective of this research was to identify the mechanism by which DNOP causes pre-neoplastic hepatic lesions; we also studied the morphological and functional changes of the mouse liver following a short-term consumption of DNOP.

2. Materials and methods

2.1. Ethical approval

All experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Augusta University Institutional Animal Care and Use Committee (IACUC).

2.2. Cell line

Two cell lines were used: AML-12 (ATCC® CRL–2254™) and FL83B (ATCC® CRL-2390™) from ATCC®. AML-12 is a normal cell line of hepatocytes from male (3 months of age) mice (*Mus musculus)* (Wu et al., 1994). AML-12 required a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 medium (GIBCO/Life technologies) with 0.005 mg/ml insulin (Cell Applications, Inc.), 0.005 mg/ml transferrin (Sigma-Aldrich Co.), 5 ng/ml selenium (Sigma-Aldrich Co.), 40 ng/ml dexamethasone at 90% (Sigma-Aldrich Co.), and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific). FL83B is a normal cell line of hepatocytes from a C57BL/6J mouse of 15–17 days of gestation (Breslow et al., 1973). FL83B required F-12 K medium with 10% FBS. All media contain 100 U/ml penicillin and 100 μg/ml streptomycin. All the cell lines were maintained at 37 °C with 5% CO_2, and the medium was changed 2 to 3 times each week. All experiments were carried out between passages 5 and 20 after receipt.

2.3. Materials

Liquid DNOP (CAS 117-84-0/Einecs 204-214-7) at a purity of ≥98% was purchased from Sigma-Aldrich (St Louis, MO). Before adding DNOP to the cells or primary culture mouse hepatocytes, DNOP was prepared by dissolving in dimethyl sulfoxide (DMSO) and then adding a medium to form a 10, 100 and 1000 ppm solution of DNOP. Control cells were given a solution with an equal amount of DMSO as DNOP-treated cells. Collagenase (CLSPA) was purchased from Worthington Biochemical Co (Lakewood, NJ). Dulbecco's minimal essential medium, fetal bovine serum, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). Leupeptin and aprotinin were purchased from Roche. The novel selective TGF-β receptor type I/II (TβRI/II) inhibitor LY2109761 was purchased from Selleckchem (Houston, TX). Protein determination reagent was purchased from Bio-Rad Life Science Research (Hercules, CA). Supersignal West Femto Chemiluminescent Substrate was purchased from Thermo Scientific (Rockford, IL). Other chemical reagents were obtained from Sigma Chemical (St Louis, MO).

2.4. ICR mice

Hsd:ICR (CD-1®) mice were provided by Envigo (Indianapolis, IN). The mice were male, 10 months old at the time of the experiment and weighed 25–30 g. Males were used because of previous reports showing a greater hepatotoxic effect of phthalates in male than in females (Barber et al., 1987; Mangham et al., 1981). Two groups of five ICR mice each were separated in cages and maintained on a 12:12 h light-dark cycle with free access to chow and water. For a month, the vehicle group was fed Purina Lab Chow soaked in 50 ml of corn oil. The DNOP-treated group was fed Purina Lab Chow soaked in a 49.95 mL of corn oil and 0.05 mL of DNOP, creating a 1000 ppm DNOP solution (Poon et al., 1997), which represents a dose equal to ~148 mg/kg/day following the dose conversion from rats to mice (Nair and Jacob, 2016). Both vehicle and DNOP diets were prepared and stored at room temperature as previously reported (Poon et al., 1997).

2.5. Antibodies

Antibodies against the following proteins were used: rabbit polyclonal antibodies to albumin (# 4929), caspase 6 (#9762), rabbit monoclonal antibodies to vimentin (D21H3)XP® (#5741), caspase 7 (#12827), bcl-2 (#2870), bim (#2933), bad (#9239), bcl-xl (#2764), phospho-histone H3 (Ser10) (#3377), mouse monoclonal antibodies to α-tubulin (#3873) and to caspase 8 (#9746) were provided by Cell Signaling Technology (Beverly, MA); goat anti-mouse IgG-horseradish peroxidase (HRP) (sc-2005) and goat anti-rabbit IgG-HRP (sc-2004) were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

2.6. Determination of rate of cell proliferation

Proliferation of AML-12 and FL83B cells was measured using Vybrant MTT cell proliferation assay kit (Molecular Probes Inc, Eugene, OR). Cells were seeded in 96-well plates at 5000 cells per well and incubated overnight to allow attachment. DNOP diluted in fresh medium was added to the cells and incubated at 37 °C at 5% CO2 for 24, 48, and 72 h. Then, the medium was removed and replaced with fresh medium without phenol red. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (10 μl) was added to each well and incubated at 37 °C for 30, 60 or 90 min 0.1% Sodium dodecyl sulfate (SDS) dissolved in 0.01% HCl solution was added to wells at 30, 60 or 90 min to stop the cell labeling. The plate was incubated at 37 °C overnight as directed in the kit. The amount of MTT that was converted to formazan by mitochondria was measured by the Gene5 program at 570 nm on the Synergy HT (BioTek Instruments, Inc.) to give optical density (OD) values. The slope of the control and DNOP-treated cells was analyzed by GraphPad Prism (La Jolla, CA) to compare differences in the rate of cell proliferation between the control (non-treated) and treatment with DNOP at each time period. Results were expressed as absorbance/hour.