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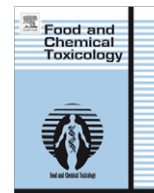

Induction of ROS, p53, p21 in DEHP- and MEHP-exposed LNCaP cells-protection by selenium compounds

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ABSTRACT

This study was designed to investigate the hypothesis that the toxic effects of di(2-ethylhexyl)phthalate (DEHP), the most abundantly used plasticizer and ubiquitous environmental contaminant that cause alterations in endocrine and spermatogenic functions in animals is mediated through the induction of reactive oxygen species (ROS) and activation of nuclear p53 and p21 proteins in LNCaP human prostate adenocarcinoma cell line. Protective effects of two selenocompounds, sodium selenite (SS) and selenomethionine (SM) were also examined. It was demonstrated that 24 h exposure of the cells to 3 mM DEHP or its main metabolite, mono(2-ethylhexyl)phthalate (MEHP, 3 μM) caused strongly amplified production of ROS. Both SS (30 nM) and SM (10 μM) supplementations reduced ROS production, and p53 and p21 activation that induced significantly only by MEHP-exposure. The overall results of this study indicated that the induction of oxidative stress is one of the important mechanisms underlying the toxicity of DEHP and this is mainly through the effects of the metabolite, MEHP. Generated data also emphasized the critical role of Se in modulation of intracellular redox status, implicating the importance of the appropriate Se status in cellular response against testicular toxicity of phthalates.

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1. Introduction

The tumor suppressor protein p53 is a transcription factor controlling cell cycle progression, cell survival, and DNA repair in cells exposed to genotoxic as well as non-genotoxic stresses (Hainaut and Hollstein, 2000; Pluquet and Hainaut, 2001). p53 is constitutively expressed in a latent form in most cells and tissues. Exposure to DNA-damage induces p53 to accumulate in the nucleus in an active form with high affinity for specific DNA sequences, after post-translational modifications at both N and C terminus of the protein (Pluquet and Hainaut, 2001). Activated p53 binds to DNA and regulates the transcription of several sets of target genes, including effectors of the cell cycle (p21/WAF1/Cip1, 14-3-3s, GADD45), apoptosis (Bax1, CD95/APO-1/FAS, AIP1), and DNA repair (p53R2) (Hainaut and Hollstein, 2000; Vousden and Lu, 2002; Fei and El-Deiry, 2003; Hofseth et al., 2004).

p21 was discovered as a "senescent cell-derived inhibitor", binds to the G_1–S/CDK (G_1–S/cyclin-dependent kinase, CDK2) and S/CDK complexes, the molecules important for the G_1/S transition in the cell cycle. p21 inhibits the activities of these molecules, and thus functions as a regulator of cell cycle progression at G_1. The expression of p21 is tightly controlled by p53, through which the p53 protein mediates the p53-dependent cell cycle G_1 phase arrest in response to a variety of stress stimuli (Harper et al., 1993; Gartel and Radhakrishnan, 2005).

Oxidative stress and reactive oxygen species (ROS) are known to play important roles in many physiological processes (Ames, 1999; Halliwell and Cross, 1994). In contrast, several studies have provided evidence that free radical-induced oxidative damage of cell membranes, DNA and intracellular proteins might be the cause of several degenerative diseases, including cancer (Barnham et al., 2004). The activation of tumor suppressor gene, p53, by a variety of cellular responses including DNA damage pathway induced by ROS has received high importance in the last decade (Ozturk et al., 2009). Several environmental chemicals, including phthalates,

Abbreviations: CM-H_2DCFA, 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DAB, 3,3′-diaminobenzidine; DCF, 2′,7′-dichlorofluorescein; DEHP, di(2-ethylhexyl)phthalate; DR5, death receptor 5; FBS, fetal bovine serum; FCS, fetal calf serum; GPx, glutathione peroxidase; MEHP, mono(2-ethylhexyl)phthalate; NAC, N-acetylcysteine; PBS, phosphate buffered saline; PP, peroxisome proliferator; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; Se, selenium; SM, selenomethionine; SS, sodium selenite; TrxR, thioredoxine reductase.

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have been shown to induce apoptosis and senescence in the reproductive tract of rodents through p53 induction (Parmar et al., 1995; McKee et al., 2006).

Di(2-ethylhexyl)phthalate (DEHP), a phthalate derivative and a well-known peroxisome proliferator (PP), is widely used as a plasticizer in the manufacture of PVC plastics. Its widespread use leads to significant human exposures through contaminated foods, food packaging, or medical products (Koo and Lee, 2004; McKee et al., 2004; Silva et al., 2006). DEHP is rapidly metabolized to its major metabolite mono(2-ethylhexyl)phthalate (MEHP) in liver, and MEHP is even more toxic than the parent compound. DEHP disturbs the quality and/or quantity of sperms, induces testicular atrophy in rodents (Parks et al., 2000; Jarfelt et al., 2005; Borch et al., 2006; Erkekoglu et al., 2011), and was shown to increase p21 expression in rat testis (Ryu et al., 2007). MEHP was reported to selectively induce oxidative stress and release cytochrome c from mitochondria in germ cells, thereby inducing apoptosis of spermatocytes and causing testicular atrophy (Kasahara et al., 2002). MEHP was also shown to cause increased p53 stability and elevation of death receptor 5 (DR5) mRNA levels coincident with the increases in the levels of apoptosis in the spermatocytes of C57BL/6 mice (Ryu et al., 2007).

Numerous enzymatic and nonenzymatic antioxidants contribute to cellular protection against oxidative stress, and studies have shown that antioxidants can suppress or delay apoptosis by acting as scavengers of ROS (Zamzami et al., 1995; Ishige et al., 2001). Among other antioxidants, selenium (Se), with its several cellular forms, is involved in the modulation of intracellular redox equilibrium (Oberley et al., 2000; Steinbrenner and Sies, 2009). Low dietary Se intakes in humans are associated with health disorders including oxidative stress-related pathologies, reduced fertility and immune functions (Broadley et al., 2006), and increased risk of cancers (Clark et al., 1991). As a component of the antioxidant enzyme families of glutathione peroxidase (GPx) and thioredoxine reductase (TrxR), Se is involved in the protection of cells from intracellular ROS (Ursini et al., 1995; Mustacich and Powis, 2000). It has been shown that Se could modulate DNA repair in cells with normal p53, and TrxR is required in the reduction of p53 cysteine residues (Seo et al., 2002; Jayaraman et al., 1997).

LNCaP cell line is a good *in vitro* model for assessing the oxidative stress potential of phthalates as they express prostate specific antigen (PSA), p53 protein, peroxisome proliferator-activated receptor α (PPARα), and peroxisome proliferator-activated receptor γ (PPARγ) (Chung et al., 1992). In addition, LNCaP cells have been shown to have responsiveness to inorganic and organic Se compounds [sodium selenite (SS) and selenomethionine (SM)] treatments (Erkekoglu et al., 2010a).

Based on those information and data, this study was designed to examine whether exposure to DEHP or MEHP in LNCaP cells increase ROS production and induce p53 and p21 proteins. To investigate the possibility of protective effects of Se in organic and inorganic forms was also aimed.

2. Materials and methods

2.1. Chemicals

DEHP was obtained from Sigma–Aldrich (St. Louis, MO, USA) and MEHP was from Cambridge Isotope Laboratories (Andover, MA, USA). RPMI 1640 medium and fetal calf serum (FCS) were purchased from GIBCO (Courbevoie, France). 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFA) was purchased from Molecular Probes Detection Technologies, Invitrogen (Eugene, OR, USA). The EnVision Plus staining kit was purchased from Dako (Carpinteria, CA, USA). Primary antibody for p53 (anti-p53) was of mouse origin, monoclonal (sc-263) and was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Primary antibody for p21 (anti-p21cip1) was of mouse origin, monoclonal (OP64) and was from Calbiochem-Merck KGaA (Darmstadt, Germany). The goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody was purchased from Invitrogen Molecular Probes (Oregon, USA). All the other chemicals including DEHP, SS, SM, fetal bovine serum (FBS), Mayers hematoxylin nuclear stain and saponin from Quillaja bark were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture and treatment

LNCaP human prostate cancer cell line (lymph-node-derived-androgen-sensitive cell line, normal for cell-cycle related tumor suppressor genes p53 and retinoblastoma Rb, wild type) was a gift from Prof. Alan Diamond, University of Illinois, USA. The cells were maintained in RPMI 1640 medium containing 5% FCS, at 37 °C in a humidified incubator under 5% CO_2. For the experiments, the cells were cultured in RPMI 1640 medium with 10% FCS and 1% penicillin/streptomycin in culture flasks in the same conditions and split one-sixth dilution each week.

SS, SM, DEHP and MEHP solutions were prepared as described earlier, and the doses chosen for DEHP (3 mM) and MEHP (3 μM) were previously shown as their approximate IC_{50} values for LNCaP cells (Erkekoglu et al., 2010a).

Experiments were performed with following treatment groups: NT-C: Non-treated LNCaP cells cultured for 72 h; SS-S: LNCaP cells supplemented and cultured with 30 nM SS for 72 h; SM-S: LNCaP cells supplemented and cultured with 10 μM SM for 72 h; DEHP-T: LNCaP cells cultured with 3 mM DEHP for 24 h; SS/DEHP-T: SS-S cells cultured with 3 mM DEHP for 24 h; SM/DEHP-T: SM-S cells cultured with 3 mM DEHP for 24 h; MEHP-T: LNCaP cells cultured with 3 μM MEHP for 24 h; SS/MEHP-T: SS-S cells cultured with 3 μM MEHP for 24 h; SM/MEHP-T: SM-S cells cultured with 3 μM MEHP for 24 h.

2.3. Measurement of intracellular ROS production

Total intracellular ROS production was measured using peroxide sensitive fluorescent probe CM-H_2DCFA as described earlier (Loikkanen et al., 1998). The study was conducted in the dark, and 70–80% confluent cells were used. LNCaP cells seeded in 96-well plates with/without SS (30 nM) and SM (10 μM) were incubated at 37 °C in a humidified incubator under 5% CO_2 for 72 h. After removal of the culture media, cells were loaded with CM-H_2DCFA in phosphate buffered saline (PBS) for 30 min at room temperature. The cellular esterase activity results in the formation of the non-fluorescent compound, the 2′,7′-dichlorofluorescin (DCFH). DCFH is rapidly oxidized in the presence of ROS to a highly fluorescent 2′,7′-dichlorofluorescein (DCF). The cells were washed, then incubated with with/without DEHP (3 mM) or MEHP (3 μM) at 37 °C in a humidified incubator under 5% CO_2 for 0, 30 and 60 min. DCF fluorescence was measured with a Perkin Elmer Victor 3 1420 multiwell fluorometer (Perkin Elmer, Buckinghamshire, UK) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. After data acquisition, Wallac 1420 Manager Software was used to analyze ROS production. Background fluorescence was obtained from cell-free wells containing 5 μM DCF in 0.5 mL of PBS and subtracted from the fluorescence values found. The multiwell plate was kept in a cell culture incubator between the measurements. The exposures were repeated 3–4 times with three parallel measurements. Fluorescence values were normalized to the cell numbers. For each condition, 8-wells were used and the mean was given as a result.

2.4. p53 and p21 Evaluation by immunocytochemistry

The expressions of p53 and p21 in LNCaP cells were examined immunocytochemically using specific primary antibodies and the EnVision Plus System. LNCaP cells, treated and cultured as described above, were washed with PBS for 3 min shaking on a shaker gently, and fixed with 4% formaldehyde in PBS at room temperature. Cells were rinsed with ddH_2O once, and washed with PBS for 3 min as were done between each step, then permeabilized with PBS/0.5% saponin/0.3% Triton X-100 for 3 × 5 min on the shaker. Cells were blocked with PBS/10% FBS/0.3% Triton X-100 at 37 °C for 1 h, then PBS washed cells were incubated with diluted primary antibody [for p53 primary antibody was anti-p53, mouse origin, monoclonal (sc-263); for p21 primary antibody was anti-p21cip1, mouse origin, monoclonal (OP64)] overnight at 4 °C. HRP conjugated secondary antibody was used directly and cells were incubated at 25 °C for 30 min. Cells were again washed with 1× PBS and later with 1× PBS/2% FBS/0.3% Triton X-100 three times, and stained with 3,3′-diaminobenzidine (DAB) chromogen solution. The staining was stopped by adding ddH_2O, and then hematoxylin was used as a nuclear stain. Images were acquired with a DC490 digital camera (Leica, Wetzlar, Germany). Cells were considered to be positive when the staining was present in the nucleus. For each condition three slides were counted and the results were given as percentage of p53 and p21 nuclear stainings.

2.5. Statistical analysis

The data were expressed as mean ± standard error (SEM). Statistical significances of differences among treatment groups were determined by use of one-way analysis of variance and covariance (ANOVA), followed by Student's *t*-test using a Statistical Package for Social Sciences Program (SPSS) version 17.0. A *p*-value <0.05 was considered as statistically significant.

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