Toxicology and Applied Pharmacology 248 (2010) 52–62

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Evaluation of cytotoxicity and oxidative DNA damaging effects of di(2-ethylhexyl)-phthalate (DEHP) and mono(2-ethylhexyl)-phthalate (MEHP) on MA-10 Leydig cells and protection by selenium

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ARTICLE INFO

Article history:
Received 30 April 2010
Revised 14 July 2010
Accepted 19 July 2010
Available online 24 July 2010

Keywords:
Di(2-ethylhexyl)-phthalate
Mono(2-ethylhexyl)-phthalate
Selenium
Oxidative stress
p53
Cytotoxicity
Antioxidant enzymes
Comet assay

ABSTRACT

Di(2-ethylhexyl)-phthalate (DEHP) is the most abundantly used phthalate derivative, inevitable environmental exposure of which is suspected to contribute to the increasing incidence of testicular dysgenesis syndrome in humans. Oxidative stress and mitochondrial dysfunction in germ cells are suggested to contribute to phthalate-induced disruption of spermatogenesis in rodents, and Leydig cells are one of the main targets of phthalates' testicular toxicity. Selenium is known to be involved in the modulation of intracellular redox equilibrium, and plays a critical role in testis, sperm, and reproduction. This study was aimed to investigate the oxidative stress potential of DEHP and its consequences in testicular cells, and examine the possible protective effects of selenium using the MA-10 mouse Leydig tumor cell line as a model. In the presence and absence of selenium compounds [30 nM sodium selenite (SS), and 10 μM selenomethionine (SM)], the effects of exposure to DEHP and its main metabolite mono(2-ethylhexyl)-phthalate (MEHP) on the cell viability, enzymatic and non-enzymatic antioxidant status, ROS production, p53 expression, and DNA damage by alkaline Comet assay were investigated. The overall results of this study demonstrated the cytotoxicity and genotoxicity potential of DEHP, where MEHP was found to be more potent than the parent compound. SS and SM produced almost the same level of protection against antioxidant status modifying effects, ROS and p53 inducing potentials, and DNA damaging effects of the two phthalate derivatives. It was thus shown that DEHP produced oxidative stress in MA-10 cells, and selenium supplementation appeared to be an effective redox regulator in the experimental conditions used in this study, emphasizing the critical importance of the appropriate selenium status.

Introduction

Phthalic acid esters are the most abundantly produced plasticizers, and known as endocrine disruptors and peroxisome proliferators (PP). Their inevitable environmental exposures in humans have been suspected to contribute to the increasing incidence of testicular dysgenesis syndrome (TDS) that is a range of reproductive defects including cryptorchidism and hypospadias in newborn boys, and testicular cancer and reduced sperm quality in adult males (Swan, 2008). In fact, TDS has been shown to develop in male rats that are exposed to phthalates *in utero* (Fisher et al., 2003). Di(2-ethylhexyl)-phthalate (DEHP) is the most important phthalate derivative with its high production, use and occurrence in the environment. It is mainly used in polyvinyl chloride plastics in the form of numerous consumer and personal care products and medical devices. The typical human exposure to DEHP ranges from 3 to 30 μg/kg/day (Doull et al., 1999) but, can be exceeded in specific medical conditions reaching 1.5 mg/

Abbreviations: BCA, bicinchoninic acid assay.; CDNB, 1-chloro-2,4 dinitrobenzene.; CM-H_2DCFA, 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate.; DAB, 3,3′-diaminobenzidine.; DCFH, 2′,7′-dichlorofluorescin.; DCF, 2′,7′-dichlorofluorescein.; DEHP, di(2-ethylhexyl) phthalate.; DMEM/F-12, Dulbecco's Modified Eagle Medium (1:1) Nutrient Mixture.; DMSO, dimethyl sulfoxide.; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid.; FBS, fetal bovine serum.; GPx1, cytosolic glutathione peroxidase.; GPx4, phospholipid hydroperoxide glutathione peroxidase.; GR, glutathione reductase.; GSH, glutathione.; GSSG, oxidized glutathione.; GST, glutathione S-transferase.; H_2Se, hydrogen selenide.; hcG, human chorionic gonadotrophin.; MEHP, mono(2-ethylhexyl) phthalate.; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.; Na_2-EDTA, disodium ethylenediaminetetraacetic acid.; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.; p53, protein 53.; PBS, phosphate-buffered saline.; PP, peroxisome proliferators.; PPARα, peroxisome proliferator-activated receptor α.; PPARβ, peroxisome proliferator-activated receptor β.; PPARγ, peroxisome proliferator-activated receptor γ.; ROS, reactive oxygen species.; Se, selenium.; SEM, standard error of mean.; Sepp1, selenoprotein P.; SM, selenomethionine.; SS, sodium selenite.; TNB, 5-thio-2-nitrobenzoic acid.; TrxR, thioredoxin reductase.

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doi:10.1016/j.taap.2010.07.016

kg/day exposure in hemodialysis patients, or as high as 10–20 mg/kg/day during neonatal transfusion or parenteral nutrition (Loff et al., 2000; Kavlock et al., 2005).

The mechanisms by which phthalates and specifically DEHP exert their toxic effects in reproductive system are not yet fully elucidated. Some of the effects of phthalate are related to their anti-androgenic potential (Ge et al., 2007; Noriega et al., 2009). A peroxisome proliferator-activated receptor α (PPARα)-mediated pathway based on their PP activity (Gazouli et al., 2002), and activation of metabolizing enzymes leading to free radical production and oxidative stress have also been suggested (O'Brien et al., 2005). Although Sertoli cells were thought to be the primary targets of phthalate exposure in testis (Grasso et al., 1993), available data suggest that Leydig cells are one of the main targets (Ge et al., 2007). Leydig cells are the primary source of testosterone production in males, and differentiation of Leydig cells in the testes is one of the primary events in the development of the male body and fertility (Zhang et al., 2008). Using the MA-10 mouse Leydig tumor cell line as a model system may, therefore, offer a valuable model in studying the direct effects of environmental chemicals, particularly those of endocrine disruptors on Leydig cell function *in vitro*. MA-10 cells are by far the best characterized and more widely used lines of cultured Leydig tumor cells that were independently derived from the M5480 tumor, a hormonally responsive mouse Leydig tumor (Ascoli, 1981).

The essential trace element selenium (Se), is the important component of cellular antioxidant defense and is involved in the modulation of intracellular redox equilibrium with its some 25 forms of cellular selenoproteins, particularly with glutathione peroxidases (GPx), and thioredoxin reductases (TrxR) (Oberley et al., 2000). Se is actively involved in many fundamental biological processes ranging from immune functions to apoptosis, and protection and repair of DNA (Ganther, 1999). It is essential for the production of normal spermatozoa and thus plays a critical role in testis, sperm, and reproduction (Flohé, 2007). The major role of Se in fertility is mediated by the membrane bound phospholipid hydroperoxide glutathione peroxidase (GPx4) which is the most abundant selenoprotein in testis (Flohé, 2007; Ursini et al., 1999). Testis Se is known to be remarkably and preferentially maintained in Se deficiency. Severe and prolonged deficiency results in sterility as spermatogenesis was arrested, whereas in less severe Se deprivation reduced sperm motility leading to impaired fertilization capacity and abnormal sperm morphology were reported (Maiorino et al., 2006). On the other hand, epidemiological studies have suggested that low serum Se levels were associated with an increase in the incidence of cancer (Clark et al., 1991). The chemopreventive and chemotherapeutic mechanisms of Se still remain unclear. Protection against oxidative damage, induction of apoptosis secondary to production of reactive oxygen species (ROS), and regulation of the thioredoxin (Trx) redox system are among the many potential mechanisms proposed (Combs and Gray, 1998; Ganther, 1999; Kitahara et al., 1993) which also seem to be closely related to the roles of Se in the reproductive system.

Oxidative stress and, thus, ROS play an important role in the modulation of several important physiological functions, but also accounts for changes that can be detrimental to the cells (Dröge, 2002). ROS are shown to contribute to cellular damage, apoptosis and cell death, but also involved in regulation of gene expression by controlling signal transduction through direct participation in cell signaling, and/or modulation of cell redox state (Dalton et al., 1999; Finkel, 1998). ROS have also been suspected of being involved in the formation of testicular atrophy in phthalate-exposed rats (Kasahara et al., 2002). On the other hand, p53 tumor suppressor protein is a redox sensitive protein known to play important roles in controlling the integrity and correctness of all processes in each individual cell. Activation of p53 by ROS and exogenous DNA damages can lead to growth arrest of the cell, DNA repair induction or apoptosis (Kim et al., 2009). Several environmental chemicals including phthalates have been shown to induce apoptosis in the reproductive tract of rodents through p53 induction (Chandrasekaran and Richburg, 2005).

On the basis of these knowledge and available data, it seems useful to examine modulation of cellular redox by Se and whether Se supplementation is effective on the effects of phthalates in rat reproductive system. In the current study, MA-10 mouse Leydig cells cultured with and without Se supplementation were used as a model, and the effects of exposure to DEHP and its major metabolite MEHP on the viability, enzymatic and non-enzymatic antioxidant status, ROS production, p53 expression and DNA damage were investigated.

Materials and methods

Chemicals. MEHP was obtained from Cambridge Isotope Laboratories® (Andover, MA, USA). The protein assay kit was from Uptima Interchim® (Montluçon, France). NaOH was purchased from Carlo Erba® (Rodano, Italy). Dulbecco's modified Eagle medium (1:1) nutrient mixture (DMEM/F-12) was 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). All the other chemicals including DEHP, sodium selenite (SS), selenomethionine (SM), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), 1-chloro-2,4 dinitrobenzene (CDNB), fetal bovine serum (FBS), Mayers hematoxylin nuclear stain, and saponin from quillaja bark; colorimetric assay kits for thioredoxin reductase (TrxR), and glutathione (GSH) measurements; Cell Lytic M cell lysis reagent, protease inhibitor cocktail, were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Anti-p53, the mouse monoclonal antibody, sc-263 was obtained from Santa Cruz Biotechnology Inc® (Santa Cruz, California, USA). The goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody was purchased from Invitrogen Molecular Probes® (Oregon, USA).

Cell culture and treatment. MA-10 mouse Leydig tumor cells were a generous gift from Prof. Mario Ascoli (Department of Pharmacology, University of Iowa College, Iowa City, USA) and maintained in Waymouth medium containing 15% (v/v) heat-inactivated horse serum, and 50 μg/mL gentamicin as previously described (Ascoli, 1981). Culturing of the MA-10 cells were accomplished in DMEM/F-12 (1:1) medium supplemented with 15% horse serum and gentamicin (50 μg/ml) using gelatin-coated culture flasks, at 37 °C in a humidified incubator under 5% CO_2. For sub-cultivation cells were trypsinized, washed with sterile phosphate-buffered saline (PBS) and centrifuged at 1500 × *g* for 5 min. For the experiments only the cells of 10–12 passages were used.

SS and SM stock solutions were prepared in sterile, deionized water. DEHP (50 mM) and MEHP (100 μM) stock solution were prepared in 0.1% DMSO, and fresh dilutions were made using culture medium to achieve final concentrations ranging from 1 to 10 mM for DEHP and from 1 to 10 μM for MEHP. Cell viability measurements were performed in MA-10 cells incubated with various concentrations of DEHP or MEHP for 24 h. For the assessment of protective effect of Se, MA-10 cells supplemented with 30 nM SS or 10 μM SM were cultured for 72 h, then exposed to various concentrations of DEHP or MEHP for 24 h while continuing the Se supplementation. The doses of Se in the form of SS and SM used in this study were chosen from preliminary experiments (not shown) as concentrations do not inhibit cell growth and do not cause cytotoxicity, but result in maximal GPx1 induction after 72 h of incubation.

For the measurement of enzyme activities, ROS and total GSH levels, p53 expression, and for alkaline single-cell gel electrophoresis (SCGE, Comet assay), following treatment groups of MA-10 cells were prepared: Non-treated cells (NT-C): MA-10 cells were cultured