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Oxidative stress and phthalate-induced down-regulation of steroidogenesis in MA-10 Leydig cells

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ABSTRACT

Previous studies have shown that phthalate exposure can suppress steroidogenesis. However, the affected components of the steroidogenic pathway, and the mechanisms involved, remain uncertain. We show that incubating MA-10 Leydig cells with mono-(2-ethylhexyl) phthalate (MEHP) resulted in reductions in luteinizing hormone (LH)-stimulated cAMP and progesterone productions. cAMP did not decrease in response to MEHP when the cells were incubated with cholera toxin or forskolin. Incubation of MEHP-treated cells with dibutyryl-cAMP, 22-hydroxycholesterol or pregnenolone inhibited the reductions in progesterone. Increased levels of reactive oxygen species (ROS) occurred in response to MEHP. In cells in which intracellular glutathione was depleted by buthionine sulfoximine pretreatment, the increases in ROS and decreases in progesterone in response to MEHP treatment were exacerbated. These results indicate that MEHP inhibits MA-10 Leydig cell steroidogenesis by targeting LH-stimulated cAMP production and cholesterol transport, and that a likely mechanism by which MEHP acts is through increased oxidative stress.

1. Introduction

Epidemiological studies have shown strong correlations between exposures to endocrine disruptors, which are defined as chemicals that mimic hormones or alter their function, and testicular dysgenesis syndrome [1,2]. Thus, decline in sperm production, poor semen quality, and increased incidences of cryptorchidism, hypospadias and testicular cancer have been attributed in part to exposures to environmental antiandrogenic compounds [1,3–7]. Identifying such compounds, and determining the developmental stage(s) that they affect and their mechanism(s) of action, have become high public and research priorities [8–11].

Phthalic acid esters are used in the production of plastics [12,13]. The global production of phthalic acid esters is approximately 6.0 million metric tons per year [14]. Humans are exposed to di-(2-ethylhexyl) phthalate (DEHP), the most widely used phthalate, through ingestion (food, infant formula, and breast milk), contact with consumer products (cosmetics and toys), inhalation, and medical procedures and devices [8,9,15,16]. Typical human exposure is about 30 μg DEHP/kg/day [17]. Medical exposures are through intravenous, oral and inhalational routes [11,18]. Dialysis patients can receive an additional 36 μg/kg/day, and newborn infants undergoing transfusion receive 1–10 mg/kg/day [8,9,19–22]. Phthalates have been shown to exhibit antiandrogenic properties in men [23], and to cause developmental and reproductive toxicity in rodents [24–27]. The in vivo exposure of steroidogenic cells to DEHP, or their in vitro exposure to mono-(2-ethylhexyl) phthalate (MEHP), the major DEHP metabolite, can result in the suppression of steroid formation [28–35]. As yet, the specific components of the steroidogenic pathway that are compromised by DEHP/MEHP, and the mechanisms by which DEHP and MEHP act to cause steroidogenic suppression, remain uncertain.

MA-10 Leydig tumor cells often are used as a model of Leydig cell steroidogenic function. A major advantage of these cells is that, unlike primary cells, they do not lose steroidogenic function when

Abbreviations: DEHP, di-(2-ethylhexyl) phthalate; MEHP, mono-(2-ethylhexyl) phthalate; LH, luteinizing hormone; dbcAMP, dibutyryl cAMP; 22HC, 22-hydroxycholesterol; P5, pregnenolone; BSO, L-buthionine-sulfoximine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; GSH, glutathione; ROS, reactive oxygen species; Gsα, stimulatory G protein α subunit; IBMX, isobutyl-methylxanthine; STAR, steroidogenic acute regulatory protein.

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cultured long-term [36]. We and others reported that culturing MA-10 cells or isolated rat primary Leydig cells with MEHP resulted in changes in the expression of genes involved in regulating reactive oxygen species (ROS) balance, and in ROS generation [34,35,37]. Exposure to ROS has been reported to affect steroidogenic function in various steroid-producing cells, including adrenal, Leydig and luteal cells [38–42], and to mediate age-related decline in steroid hormone production [43]. These studies, taken together, led us to hypothesize herein that the induction of ROS by MEHP mediates the suppressive effects of MEHP on critical steps in the steroidogenic pathway, resulting in reduced steroidogenesis.

2. Materials and methods

2.1. Chemicals and reagents

MEHP (P1073) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Japan). Dibutyryl cAMP (dbcAMP), L-buthionine-sulfoximine (BSO), 22-hydroxycholesterol (22HC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Waymouth's MB/752 medium, poly-L-lysine, horse serum, cholera toxin and forskolin were from Sigma–Aldrich (St. Louis, MO). 2′,7′-Dichlorodihydrofluorescein diacetate (DCF) was from Invitrogen (Carlsbad, CA). [1,2,6,7-^{3}H(N)]-progesterone was from Perkin Elmer Life Sciences, Inc. (Boston, MA). Progesterone antibody was from ICN (Costa Mesa, CA). STAR antibody was from ABR, Inc. (Golden, CO). Stimulatory G protein α subunit (Gsα) antibody was from Enzo Life Sciences (formerly Biomol, Plymouth Meeting, PA). The HRP-conjugated anti-rabbit donkey IgG and [^{3}H]-cAMP assay kits were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). Bovine LH (USDA-bLH-B-6) was provided by the USDA Animal Hormone Program (Beltsville, MD). Pregnenolone (P5) and progesterone were from Steraloids (Newport, RI).

2.2. MA-10 cell culture

The MA-10 mouse Leydig tumor cell line was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA). The cells were cultured in Waymouth's MB/752 medium containing 15% horse serum and 5% CO_2 at 34 °C [36]. For MEHP treatment, cells with 70% confluence were incubated with the reagent (0–400 μM) for 2–24 h. In some studies, cells were pre-incubated with BSO (100 μM, 24 h) to deplete glutathione (GSH) and thus alter the intracellular redox environment, and then with increasing concentrations of MEHP. In studies in which progesterone was assayed, cells were incubated in MEHP (0–400 μM), and then switched to serum-free M-199 medium containing 0.1% BSA and incubated for 2 h with luteinizing hormone (LH, 100 ng/ml), dibutyryl cyclic AMP (dbcAMP, 2 mM), 22-hydroxycholesterol (22HC, 25 μM), or pregnenolone (P5, 25 μM). After treatment, the medium was collected and stored at −80 °C for subsequent radioimmunoassay (RIA) analysis, and the cells were lysed for Western blots. The viability of cells was estimated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (see Section 2.6).

2.3. Progesterone and cAMP assays

Medium collected at the end of each experiment was stored at −80 °C. Progesterone concentration in the medium was determined by RIA and expressed as micrograms per milligram cellular protein. For cAMP assays, the cells were cultured in a 24-well plate with MEHP (0–400 μM) for 24 h, then switched to phenol red-free M-199 medium containing LH (100 ng/ml), cholera toxin (10 μg/ml) or forskolin (500 μM) for 2 h. The medium also contained isobutyl-methylxanthine (IBMX, 100 μM) to inhibit phosphodiesterase activity. At the end of the incubation, an equal volume of Tris–HCl buffer (0.05 M, pH 7.5) containing 4 mM EDTA and 2 mg/ml theophylline was added to the culture medium to block cAMP degradation. The plates were frozen on dry ice and stored at −80 °C before cAMP and progesterone assays. cAMP levels were measured using the [^{3}H]cAMP assay system from GE Healthcare Bio-Sciences Corp (Piscataway, NJ) according to the manufacturer's instructions. The sensitivity of the assay was 0.05 pmol per assay tube.

2.4. Glutathione (GSH) assay

GSH was measured as previously described [44], with minor modifications. In brief, cells were lysed in 5% metaphosphoric acid and sonicated. Protein was precipitated by centrifugation at 13,000 × *g* for 30 min. The supernatant was diluted 10 times with sodium phosphate buffer (0.1 M, pH 8.0, with 5 mM EDTA). Diluted sample (10 μl) was incubated with 10 μl of o-phthalaldehyde (in methanol) and 180 μl of phosphate buffer for 15 min at room temperature. Fluorescence was read with a BioRad luminescence spectrometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The cellular GSH content was calculated using a concurrently run GSH standard curve.

2.5. Analysis of intracellular ROS concentrations

Intracellular ROS concentration was assessed by measuring the ability of the cells to oxidize the redox sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (DCF). In brief, cells were cultured in black 96-well florescence assay plates (BD Falcon, Franklin Lakes, NJ). After their preincubation with or without BSO for 24 h, the cells were treated with MEHP (0–400 μM) with or without the antioxidant vitamin E (40 μg/ml) for 2 h. The cells then were incubated in 200 μl M199 medium containing 20 μM DCF for 30 min. After washing 3 times with PBS, 50 μl PBS was added, and the plates were read on a DTX800 Multimode Detector (Beckman Coulter, Fullerton, CA) with excitation of 485 nm and emission of 535 nm. DCF fluorescence also was examined by fluorescence microscopy. The cells were cultured in poly-L-lysine-coated six well chamber slides, treated with MEHP, and then incubated with DCF as above. Images were obtained with a Nikon Eclipse 800 microscope equipped with a Princeton Instruments 5-Mhz cooled CCd camera, custom CRI color filter, and IP-Lab digital image analysis software (Scanalytics Inc., Fairfax, VA). All photos were taken at the same magnification and with the same exposure times.

2.6. Cell viability assays

Cell viability was estimated by MTT reduction, mitochondrial membrane potential, or plasma membrane permeability. The MTT assay measures the reduction of MTT to blue formazan in viable cells [45]. After their incubation with MEHP (up to 400 μM, 24 h), cells were incubated with MTT (0.5 mg/ml) for 1 h. The medium was then removed and reduced formazan was dissolved in 100 μl acidified (0.04 N HCl) isopropanol at room temperature for 20 min. The dissolved formazan then was transferred to a new 96-well plate and read by a DTX800 Multimode Detector (Beckman, Coulter, Inc., Fullterton, CA) at 562 nm wavelength. Control (blank) wells contained cells without MTT but with isopropanol. Cells from three different experiments were analyzed for each treatment.

2.7. Western blot analysis

Gsα, STAR and β-actin proteins were analyzed by Western blots. Gsα was analyzed after treatment of cells with MEHP (0–400 μM) for 24 h. For STAR and β-actin protein analysis, the MEHP treated cells were further stimulated with LH (100 ng/ml) for 2 h. Thirty