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Long -term maintenance of luteinizing Hormone–Responsive testosterone formation by primary rat Leydig cells in vitro

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ABSTRACT

The inability of cultured primary Leydig cells to maintain luteinizing hormone (LH)-responsive testosterone formation in vitro for more than 3–5 days has presented a major challenge in testing trophic effects of regulatory factors or environmental toxicants. Our primary objective was to establish culture conditions sufficient to maintain LH-responsive testosterone formation by Leydig cells for at least a month. When isolated rat adult Leydig cells were cultured in DMEM/F12 and M199 culture medium containing insulin (10μg/ml), PDGFAA (10 ng/ml), lipoprotein (0.25 mg/ml), horse serum (1%) and a submaximal concentration of LH (0.2 ng/ml), the cells retained the ability to produce testosterone in vitro for at least 4 weeks. By using the longer-term culture conditions of this system, we were able to detect suppressive effects on testosterone production by low levels of the toxicant MEHP (mono-(2-ethylhexyl) phthalate), an active metabolite of the plasticizer DEHP, that were not detected by short-term culture.

1. Introduction

The primary function of testicular Leydig cells is to produce the male hormone, testosterone (T), which is essential for the development of the male reproductive system and for the maintenance of numerous reproductive functions (Nef and Parada, 2000; Smith and Walker, 2014). Development of methods by which to isolate and culture Leydig cells has allowed short-term studies of their regulation and function in vitro. However, longer-term in vitro studies of the effects of such factors as regulatory molecules, chronic exposures to environmental toxicants, or the effects of genetic manipulation have been limited by an inability to maintain LH-responsive, testosterone-producing Leydig cells in vitro for more than 3–5 days (Klinefelter and Ewing, 1989; Risbridger and Hedger, 1992). Given these limitations, many studies have been conducted using tumor cell lines (MA10, R2C, TTE-1, BLT-1, MT3, I-10, MLTC-1) as alternatives to primary cells (Odermatt et al., 2016). Although these cell lines can stably produce steroids in vitro for long periods of time, they are not optimal as they typically are derived from tumors and often have modified steroidogenic pathways. For example, many among these cells produce progesterone as their final product, not testosterone (Ascoli, 1981; Odermatt et al., 2016); and some have lost the ability to respond to LH stimulation (Freeman, 1987). These limitations make it apparent that the development of ways to maintain primary Leydig cell steroidogenic function long-term, in vitro, would be desirable.

The establishment of a culture system for the long-term maintenance of LH-responsive testosterone production by primary Leydig cells would be particularly desirable in the toxicology field. Many among the environmental toxicants that affect the reproductive system target testosterone production by Leydig cells. Animal experiments typically are conducted to test the toxicity of given compounds. In 2007, however, the U.S. National Research Council (NRC) released a report entitled “Toxicity Testing in the 21st Century: A Vision and a Strategy” that called for a shift from whole-animal testing to the use of in vitro methods using cells, cell lines, or cellular components (National Research Council (NRC), 2007). Included in the rationale given for suggesting this approach was that in vitro methods would reduce animal pain and distress (National Research Council (NRC), 2007; Gibb,

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2008). However, it was recognized that a significant drawback of cell-based studies using freshly isolated cells was that testing could be done only for short periods of time, whereas animals and humans are exposed for far longer periods. This provides additional rationale to develop methods to maintain Leydig cell testosterone production in vitro for weeks rather than days so as to enable the examination of chronic effects of potential toxicants under highly controlled conditions.

In the present study, we describe a modified culture medium that makes it possible to culture freshly isolated rat Leydig cells that produce testosterone in response to LH for at least 4 weeks. Although there was a decline in the capacity of the cells to produce testosterone during the incubation period, the cells nonetheless maintained their responsiveness to LH and other steroidogenic stimulators comparably to freshly isolated cells. We then used this system to examine the effects of mono-(2-ethylhexyl) phthalate (MEHP), a common phthalate metabolite, on testosterone production. We report a negative effect of chronic exposure to low MEHP concentrations that were not detectable by short-term exposures.

2. Materials and methods

2.1. Chemicals

The culture media (M-199, DMEM/F12) and the Click-iT EdU (5-ethynyl-2′-deoxyuridine) kit were from Invitrogen (Carlsbad, CA). Human insulin, ITS supplement (10 μg/ml human insulin, 5.5 μg/ml human transferrin, and 5 ng/ml sodium selenite) and horse serum were from Sigma-Aldrich (St. Louis, MO). High-Capacity cDNA Reverse Transcription kit and TaqMan Gene Expression assay were from Applied Biosystems (Waltham, MA). Type I collagenase was from Worthington (Lakewood, NJ). Trypan blue solution (0.4%) was from Thermo Fisher Scientific (Waltham, MA). Bovine LH (USDA-bLH-B-6) was provided by USDA Animal Hormone Program, Beltsville, MD). [1,2,6,7,16,17-^{3}H(N)]-testosterone (115.3 Ci/mmol) was from PerkinElmer Life Sciences, Inc. (Boston, MA). The other reagents and chemicals used in this study are summarized in Table 1.

2.2. Animals

Male Brown Norway rats of ages 4–6 months were obtained through the National Institute on Aging, supplied by Harlan Sprague Dawley, Inc. (Indianapolis, IN). Rats were housed in controlled light (14-h light, 10-h dark) and temperature (22 C), and had free access to rat chow and water. All procedures were in accord with the NIH Guide for Care and Use of Laboratory Animals, with protocols approved by the Johns Hopkins Animal Care and Use Committee.

Table 1
Reagents or chemicals used in the experiments.

Reagent	Concentration (range tested)	Manufacturer
T3	50 nM (10–250)	Sigma-Aldrich
Insulin	10 μg/ml (0–10)	Sigma-Aldrich
SAG	0.5 μM (0.05–5)	EMD Bioscience
Li	5 mM (0.5–50)	Sigma-Aldrich
PDGFAA	10 ng/ml (0–10)	ProSpec
Lipo	0.25 (0.25–1)	Sigma–Aldrich
HS	1% (1–5%)	Sigma–Aldrich
Ovine LH	0.2 ng/mL (0–10)	Hormone Program USDA
dbcAMP	1 mM	Sigma-Aldrich
22HC	12.5 μM	Sigma–Aldrich
P5	12.5 μM	Steraloids
P4	12.5 μM	Steraloids
A	5 μM	Steraloids
MEHP	100 μM (0–300)	Tokyo Kasei Kogyo Co

2.3. Leydig cell purification

Leydig cells were isolated by a combination of Percoll and BSA density gradient centrifugation, as previously described (Salva et al., 2001). In brief, the testes were decapsulated and digested in dissociation buffer (M-199 medium with 2.2 g/L HEPES, 1.0 g/L BSA, 2.2 g/L sodium bicarbonate) containing collagenase I (0.5 mg/ml) at 34 ˚C, with slow shaking (90 cycles/min, 30 min). To separate the interstitial cells from the seminiferous tubules, digested testes were placed in a solution containing 1% BSA for one minute. The supernatants were collected and the interstitial cells were pelleted by centrifugation (1500 g, 5 min). Leydig cells were purified by Percoll gradient separation (55% Percoll, 27,000 g, 1 h) and then by BSA gradient centrifugation (0–10% BSA, 450 g, 10 min). The final purity of the Leydig cells obtained, determined by staining the cells for 3β-hydroxysteroid dehydrogenase (HSD3b) activity, was about 95%.

2.4. Culture of isolated Leydig cells in vitro

The development of long-term (2 or 4 week) culture conditions for Leydig cells involved stepwise modification of a procedure described previously for short-term culture (Klinefelter and Ewing, 1989). Briefly, purified Leydig cells were suspended in a mixture of M199 and DMEM/F12 (volume 1:1) culture medium supplemented with 2.2 g/liter NaHCO3, 2.4 g/liter HEPES, 0.1% BSA, and Penicillin: Streptomycin (100IU:100 μg/ml), pH 7.4. The cells were cultured in 96-well (about 10^5 cells/well) or 24-well (about 5×10^5 cells/well) culture plates in the presence of increasing concentrations of LH (0–10 ng/ml) and added growth factors. At the end of the culture period, the cells were challenged with maximally stimulating LH (10 ng/ml) for 24 h, and testosterone in the media was assayed by radioimmunoassay (RIA). Leydig cell purity was assayed by counting HSD3b or CYP11A1 positive cells before their culture and at weeks thereafter. Leydig cell viability was estimated by trypan blue exclusion. The cells were incubated with medium containing 0.1% trypan blue solution (5 min) and the blue-colored cells were counted.

2.5. CYP11A1 immunofluorescence and 3βHSD activity staining

After their culture, cells were washed with Ca^{2+}- and Mg^{2+}-free HBSS solution, fixed in neutral buffered formalin, and after washing with HBSS solution, incubated with CYP11A1 primary antibody (1:100; Rabbit IgG, Chemicon International) overnight. Cells were then treated with fluorescent secondary antibodies (Alexa-conjugated anti-rabbit IgG, 1:1000) for 1 h and, after washing, examined using a Nikon Eclipse 800 microscope. Cell nuclei were visualized by staining DNA with 0.3 μM DAPI. Staining for HSD3b was conducted as previously reported (Klinefelter and Ewing, 1989; Stanley et al., 2012). In brief, cells were washed in HBSS solution and then dried on slides at room temperature for 30 min. Slides were stained for 40 min with a solution containing 0.4 mM 5β-androstan-3β-ol-17-one steroid substrate, 1 mg/ml NAD, and 0.2 mg/ml tetranitro blue tetrazolium. After washing in HBSS solution, the stained tissue was fixed with 10% formalin in HBSS containing 5% sucrose (15 min).

2.6. Cell proliferation

Cell proliferation was assessed with the Click-iT EdU imaging kit from Invitrogen (Odeh et al., 2014). Dividing cells were labeled with EdU (10 μM, 24 h) after their treatment with or without LH plus four factors for 1 d or 7 d. All cells were visualized by DAPI (4′,6-diamidino-2-phenylindole) staining of the nucleus. The labeled nuclei were visualized under a Nikon Eclipse 800 microscope (excitation/emission at 495/519 nm) and quantified. For some studies, Leydig cells were identified by co-staining of the EdU-labeled cells with CYP11A1 antibody. EdU-labeled cells were counted and expressed as the proportion