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Deregulation of the *Sod1* and *Nd1* genes in mouse fetal oocytes exposed to mono-(2-ethylhexyl) phthalate (MEHP)

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ABSTRACT

Mono-(2-ethylhexyl) phthalate (MEHP) is the active metabolite of the diester (DEHP), a well-known reproductive toxicant. Since, different molecular mechanisms underlying this toxicity are not well understood, the effects of MEHP on cell viability and gene expression were assessed in murine fetal oocytes cultured *in vitro*. Oocyte survival decreased significantly after a 24 h exposure to MEHP and hence, the effects of MEHP on changes in gene expression were analyzed using cDNA libraries and differential screenings. In these assays, the genes that suffered the most severe deregulation corresponded to those encoding Cu–Zn superoxide dismutase (*Sod1*) and a mitochondrial respiratory chain protein (*Nd1*). Indeed, functional assays on somatic cells transfected with a *Sod1* luciferase reporter construct demonstrated its specific MEHP dose-dependent up-regulation, confirming that the expression of this gene is deregulated in response to MEHP.

1. Introduction

Phthalates are compounds widely used as plasticizers, solvents, additives, lubricants and insect repellent. Each of these compounds has different levels of toxicity and they are known to cause reproductive disorders [1]. Approximately 3.5 million tons of the main phthalate, di-(2-ethylhexyl) phthalate (DEHP), are produced annually worldwide [2] and indeed, DEHP is considered a ubiquitous environmental contaminant [3]. The general exposure of humans to DEHP was estimated as 30 μg/kg-day, primarily from residues in food [4]. Nevertheless, occupational and clinical exposure may increase this level to between 0.5 and 5 mg/kg-day [5]. Among all types of phthalates, DEHP is considered to be one of the most potent compounds causing adverse effects on reproduction and development in animal studies. In most species, DEHP is rapidly hydrolyzed to 2-ethylhexanol (2-EH) and mono-(2-ethylhexyl) phthalate (MEHP), the latter constituting the active toxic metabolite [6].

DEHP has been shown to be genotoxic in human mucosa cells and lymphocytes [7], as well as producing carcinogenic effects [8], peroxisome proliferation and liver tumors in rodents [9]. Certain teratogenic effects of MEHP have also been reported in animals [10]. However, the effects of DEHP in the reproductive system have been considered of special relevance due to their recognized activity as endocrine disruptors [11]. In fact, both *in vivo* and *in vitro* studies have demonstrated that DEHP and MEHP have estrogenic [12] and mainly antiandrogenic effects [13].

Testicular toxicity of DEHP on animals has been widely reported, and studies in rats and mice have shown that DEHP causes dose-dependent atrophy of the seminiferous tubules and testes [14–16]. In the testis, the Leydig and Sertoli cells are thought to be the target cells of phthalate toxicity [17–20]. In the ovaries, MEHP alters the metabolism of cells that provide support for oocyte growth and differentiation (i.e. granulosa cells). Exposure of adult, regularly cycling, female rats to DEHP for 8 days suppresses the production of preovulatory follicle granulosa cell estradiol. This suppression of serum estradiol causes secondary increases in FSH levels and impedes the LH surge necessary for ovulation. Consequently, ovulation is altered in rats exposed to DEHP [21]. Ovaries cultured *in vitro* and treated with DEHP also show abnormal profiles of steroid production [22]. In addition, bovine oocytes exposed to MEHP *in vitro* at similar concentrations to those found in the blood of patients after a transfusion, negatively modulated the meiotic progression of oocytes [23].

We have previously used a model system of cultured fetal oocytes to study the genetic effects of pharmacological compounds on the development of oocytes *in vitro* [24]. Considering the potential exposure to phthalates during embryonic development, we

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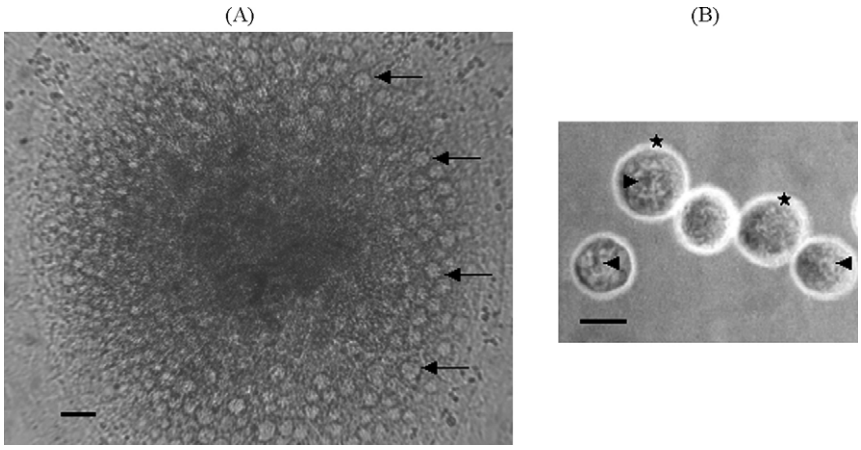

Fig. 1. *In vitro* culture of fetal oocytes. Fetal ovary explants were incubated in tissue culture vessels in 250 μl Waymouth culture medium. After 6 days in culture, hundreds of oocytes (arrows) had grown out of each ovarian explant (A). Bar, 100 μm. For gene expression analysis, oocytes were isolated from the ovary explants by mechanical dissociation with a pipette. The germinal vesicle (arrowheads) and zona pellucida (stars) were clearly seen (B). Bar, 50 μm.

examined the effects of the active metabolite, MEHP, on mouse fetal oocyte development *in vitro*. To further understand the potential molecular mechanisms by which DEHP could initially affect growing oocytes, the changes in gene expression provoked by MEHP were assessed by the differential screening of cDNA libraries. The levels of expression of differentially expressed genes were validated in MEHP-exposed oocytes by real time RT-PCR to quantify the extent of the transcriptional control. Finally, functional assays to study the effects of MEHP on transcriptional regulation were performed by using reporter constructs containing the promoter of the Cu–Zn superoxide dismutase (*Sod1*) gene, the activity of which was significantly up-regulated in oocytes exposed to MEHP.

2. Materials and methods

2.1. In vitro culture of fetal oocytes and MEHP treatment

All procedures involving the use of mice were performed under the regulations established by our Institutional Bioethics Committee on animal care and in accordance with the European Council Directive 86/609/EEC. Fetal ovaries were dissected out from CD-1 mouse embryos 17 days post-coitum and each ovary was cut into small fragments (0.5–1 mm) with fine needles (G 5/8) under a stereomicroscope. These ovarian explants were cultured in Waymouth medium (Gibco) supplemented with 5% horse serum, 2.5% fetal calf serum (FCS) (both heat inactivated) and 100 μg/ml penicillin–streptomycin, as described previously [24,25]. After 6 days in culture (Fig. 1), when most of the oocytes had reached the germinal GV stage, the cultures were exposed for 24 h to different concentrations of MEHP diluted in DMSO (125, 250 and 500 μM) to evaluate cell survival. The cells were then washed with fresh medium and oocyte survival was evaluated after a further 0, 4, 9 and 14 days in culture by examining their uptake of the supravital stain Hoechst 33258 over 20 min at 37 °C. In the control cultures, the cells were exposed to 0.05% DMSO (v/v) alone in the medium, equivalent to the concentration used in the MEHP experiments.

To assess the specific responses of the oocytes to MEHP rather than their general cytotoxic response, gene expression was analyzed using live oocytes that showed no morphological effects after a 24 h exposure to MEHP (250 μM). Live oocytes were isolated from the cultured explants of fetal ovaries by manually dissociating the tissue with a pipette until they were released (Fig. 1B). These oocytes were then washed in PBS–DEPC and stored in PCR tubes at −70 °C until they were analyzed (10 oocytes per tube in 0.5 μl PBS).

2.2. Library construction and cDNA probes

cDNA's were obtained by reverse transcription (RT) of the mRNA isolated from 10 oocytes after they were lysed by two rapid thawing and freezing steps. The RT, polyA tailing of cDNAs and subsequent amplification of cDNAs (in range size between 400 and 900 bp) were performed directly on the cell lysates following the methods described in [24]. The cDNAs obtained from MEHP-exposed and control oocytes were used to generate both the cDNA libraries and probes to be used in the differential screening.

The cDNA libraries from control and MEHP-exposed oocytes were generated in Lambda-ZapIII vector (Stratagene) [24]. The cDNA library from control cells had a titer of 1.5×10^6 pfu/ml (98% recombinant phages) and that of the MEHP-exposed cells was 3.7×10^6 pfu/ml (96% recombinant phages). Both libraries were amplified to the order of 10^9 pfu/ml and analyzed by electrophoresis, and the sequence of randomly selected clones revealed no redundancy. The library from the control oocytes was used in this work as bait for the differential screening to select genes deregulated by MEHP. The library from MEHP-exposed oocytes was stored in order to be used in further experimental work.

2.3. Selection of cDNA clones by differential screening

Plaques were transferred to nitrocellulose membranes in duplicate at a density of 2000 pfu/plate. One of these sets of membranes was incubated with the α^{32}P-dCTP labeled cDNA probes obtained from the untreated control oocyte mRNAs, while the other was incubated with the similarly labeled cDNA probes from the MEHP-exposed oocytes. Differentially expressed genes from 10,000 screened plaques were detected by autoradiography, and the clones identified were amplified by PCR using primers corresponding to the flanking cDNA inserts in the vector. These amplified cDNAs were resolved by electrophoresis, and transferred onto membranes in duplicate to perform a second differential analysis under the same conditions. Clones were only considered as genuinely positive when clear differential signals appeared in the second screening.

2.4. Nucleotide sequencing and analyses of sequence homologies

Positive plaques were excised to release the pBluescript phagemid (Stratagene) and the corresponding cDNAs were sequenced using an automatic sequencer. Sequence homologies were analyzed with the Basic Local Alignment Search Tool (BLAST) algorithm [26].

2.5. Quantification of gene expression by real time RT-PCR

Two genes identified as differentially expressed in the screens, *Sod1* and the NADH-ubiquinone oxidoreductase chain 1 (*Nd1*), were analyzed by real time RT-PCR using a high-speed thermal cycler (LightCycler™; Roche Diagnostics) to quantify the transcriptional control of these genes in oocytes exposed to MEHP (250 μM) [27]. The results were normalized to the expression of ribosomal protein S16 housekeeping gene (S16). To further evaluate the specificity of *Sod1* regulation by MEHP in oocytes, the expression of *Sod1* in oocytes exposed to another known reprotoxicant, N′-ethyl-N′-nitrosourea (ENU; a concentration of 1 mM was used since it affected oocyte viability to a similar extent as 250 μM MEHP) [28], was also evaluated by qRT-PCR. RT reactions were performed as indicated above on DNA digested for 5 min at 37 °C with 2 U of RNase-free DNase prior to the RT reaction to avoid genomic DNA amplification.

The primers used for PCR were designed using the Primer3 program [29] as follows: SOD1 U (5′-TATGGGGACAATACACAAGG-3′), SOD1 L (5′-GTTTGAGGGTAGCAGTTGAG-3′), ND1 U (5′-GCTTTACGAGCCGTAGCCGT-3′), ND1 L (CGGTTTGTTTCTGCTAGGGT-3′), S16 U (5′-AGGAGCATTTGCTGGTGTGGA-3′), S16 L (5′-GTCACCAGGCCTTTGAGATGGA-3′).

The PCR and melting curve determinations were performed using the DNA master SYBR Green I kit as recommended by the manufacturer (Roche) and 2 μl of the oocyte cDNA was used for amplification (equivalent to 2 oocytes). A standard concentration curve was produced by amplifying S16 from serial dilutions (1:100, 1:1000 and 1:5000) of mouse testis cDNA. After an initial denaturation at 94 °C for 2 min, PCR was carried out over 40 cycles with denaturation at 94 °C for 1 s, annealing at 61 °C for 10 s and extension at 72 °C for 16 s, with a ramping time of 20 °C/s. To assess the specificity of the PCR products amplified, melting curves were gener-