Human testis steroidogenesis is inhibited by phthalates

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**Background:** Phthalic acid esters are widely used in the manufacture of plastics. Numerous studies have shown that these phthalates impair testicular testosterone production in the rat. However, the scarce and contradictory data concerning humans have cast doubt over whether these compounds are also anti-androgenic in man. We therefore investigated the direct effects of di-(2-ethylhexyl) phthalate (DEHP) and mono-(2-ethylhexyl) phthalate (MEHP) on organo-cultured adult human testis and a human cell line.

**Methods:** Adult human testis explants or NCI-H295R adrenocortical human cells were cultured with DEHP or MEHP. The effects of ketoconazole, used as a reference molecule, were also assessed.

**Results:** In both models, DEHP and MEHP significantly inhibited testosterone production. The effects of both phthalates appeared to be specific for steroidogenesis, as INSL3 production by Leydig cells was not altered. Furthermore, the phthalates of interest had no effect on inhibin B production by Sertoli cells or on germ cell apoptosis. As only a small fraction of the phthalates added was found in the testis explants, and as these compounds were found to be metabolized, we estimate that the anti-androgenic effects observed occurred at concentrations of phthalates that are of the same order of magnitude as exposures reported in the literature for men.

**Conclusions:** We provide the first evidence that DEHP and MEHP can inhibit testosterone production in the adult human testis. This is consistent with recent epidemiological findings of an inverse correlation between exposure to MEHP and testosterone concentrations.

**Key words:** human testis / testosterone / phthalates / endocrine disruption / steroidogenesis

**Introduction**

Phthalates are man-made chemicals widely distributed in the human environment. Numerous studies have been performed to assess the effects of phthalates, principally on the male reproductive functions of the fetal and prepubertal rat. These experiments have led to the conclusions that exposure to phthalate esters results in the inhibition of testicular testosterone synthesis (Parks et al., 2000) associated with the development of abnormalities of the reproductive tract (Imajima et al., 1997; Gray et al., 2000; Parks et al., 2000; Shono et al., 2005; Lin et al., 2008). In post-pubertal rats, phthalates were also found to decrease sperm production (Barlow et al., 2004).

Studies on the effects of phthalates in human are few and often contradictory. The concentrations of phthalates in mother’s urine have been found to be associated with a shorter anogenital distance in newborn boys (Swan et al., 2005; Swan, 2008), but such an association was not found by others (Huang et al., 2009). Significant correlations between phthalate monoesters in breast milk samples and several infant hormones have also been demonstrated (Main et al., 2006). However, two recent in vitro studies did not find any suppressive effect of mono-(2-ethylhexyl) phthalate (MEHP) or of mono-n-butyl phthalate (MBP) on fetal testosterone levels (Hallmark et al., 2007; Lambrot et al., 2009). Furthermore, a recent study in healthy adult men has failed to demonstrate any association between MEHP and reproductive biomarkers (Jonsson et al., 2005).
In contrast, Pan et al. (2006) reported that workers exposed to MBP and MEHP had significantly lower free testosterone levels than unexposed men. A more recent study also showed that urinary MEHP concentrations were negatively correlated with free testosterone and estradiol levels in infertile men (Meeker et al., 2009). The latter observations argue in favour of phthalates having anti-androgenic effects in the adult human testes. However, no direct effect on human testicular steroidogenesis has yet been shown.

Here, we report a study investigating the potential direct effects on human testicular function of both di-(2-ethylhexyl) phthalate (DEHP), the most commonly used and widely distributed phthalate, and its major metabolite, MEHP.

Materials and Methods

Organotypic cultures of human testis explants (TETAX)

The study protocol was approved by the local ethics committee, and informed consent was obtained from all donors. Testes were obtained from prostate cancer patients and processed as previously described (Roulet et al., 2006). Treatments involved culturing two testis explants of about 3 mm³ onto a PET insert (Falcon Labware; Becton Dickinson, Lincoln Park, NJ, USA) at the interface of air in 1 ml medium (Dulbecco’s modified Eagle’s medium; Sigma-Aldrich, Saint Quentin Fallavier, France) containing 0.1% dimethylsulphoxide (DMSO) as a control, or 10⁻³–10⁻⁴ M of DEHP (purity > 99%; Interchim, Montluçon, France) or of MEHP (purity > 99%; Interchim) in 0.1% DMSO. Ketoconazole (Sigma-Aldrich) was also used at 10⁻⁵ M in 0.1% DMSO. For each condition, a minimum of four wells (i.e. eight explants) was examined. This culture system was incubated for 24 or 48 h in a humidified atmosphere containing 5% CO₂ at 34°C. The culture media were fully collected after either 24 or 48 h and stored at −20°C. On the day of collection, four testis explants were fixed in either neutral buffer 4% formaldehyde or Bouin’s fixative for 2 h at +4°C, embedded in paraffin, sliced into 5.0 μm thick sections and stored at +4°C until immunostaining.

Immunostaining

Immunohistochemistry with formaldehyde-fixed, paraffin-embedded tissues was used to estimate the number of cells undergoing apoptosis. Testis explant sections (5 μm thick) were deparaffinized, rehydrated, antigen-retrieved for 20 min at +90°C in boiled EDTA buffer (pH 6), and washed three times in 0.05 mol/l phosphate-buffered saline (PBS, pH 7.6). Endogenous peroxidases were inactivated with a 5 min treatment in PBS–3% H₂O₂. Non-specific binding sites were blocked in PBS supplemented with 0.1% Tween 20, 2% bovine serum albumin and 0.1% DMSO or 10⁻² M ketoconazole, or 10⁻³–10⁻⁴ M of DEHP or MEHP in 0.1% DMSO. The digested explants were dissolved in 10 ml Ultima Gold scintillating液 (PerkinElmer, Courtaboeuf, France). The methylthiazolyldiphenyl-tetrazolium bromide (MTT) test was performed on freshly isolated germ cells. Cell viability was assessed on tissue sections or cell suspensions using a MTT reduction assay (MTS, Promega). 1.500 μl of each incubation medium and explant or cell homogenates was measured by counting Using a Packard TriCarb 4430 TR scintillation counter (PerkinElmer).

Cultures of the NCI-H295R cell line and treatment (CELIAS)

The NCI-H295R human adrenocortical cell line, which expresses steroidogenic enzymes, has previously been used to study anti-androgenic compounds (Sanderson et al., 2000; Gracia et al., 2006) and was generously provided by Dr Feige (Inserm U878, Grenoble, France). Cells were grown for 1–10 passages in 75 cm² flasks with 10 ml of supplemented medium at 37°C in a 5% CO₂ atmosphere. The supplemented medium was RPMI medium 1640 (Sigma-Aldrich) with 1% ITS plus (Becton Dickinson, Le Pont de Claix, France), 2% fetal bovine serum (Eurobio) and antibiotics. The cells were then sub-cultured at a density of 10⁶ cells/well in a 12-well culture plate, and were incubated for 5 days. The medium was then replaced with serum-free RPMI 1640 containing 0.1% bovine serum albumin and 0.1% DMSO or 10⁻³ M ketoconazole, or 10⁻³–10⁻⁴ M of DEHP or MEHP in 0.1% DMSO and the culture was continued for 24 or 48 h. The media were collected and stored at −20°C. No DEHP, MEHP or ketoconazole-induced cytotoxicity was observed using the methylthiazolyldiphenyl-tetrazolium bromide cell viability assay (Denizot and Lang, 1986).

DEHP and MEHP distribution in the TETAX

The explants were incubated with 10⁻³ M radiolabelled DEHP or MEHP as previously described (Chauvigne et al., 2009). We used ring UL ¹⁴C-DEHP (244.2 MBq/mmol; purity > 98%; Sigma-Aldrich) or ¹⁴C-MEHP (432.9 MBq/mmol) obtained by incubating radiolabelled DEHP (1.5 KBq) with esterase from porcine liver (2.9 Units) in 0.5 ml 0.1 M Tris buffer (pH 7.4) for 30 min under shaking at 37°C. At the end of the culture, explants were separated from filters and both were digested in 1 ml Soluene-350 (PerkinElmer, Courtaboeuf, France) and then incubated at 55°C (2 h for the testes, overnight for the filters). The digested explants were dissolved in 10 ml Ultima Gold scintillating liquid (PerkinElmer), and radioactivity was counted using a TriCarb 2100 TR scintillation counter (PerkinElmer).

DEHP and MEHP standards were purchased from Fluka/Riedel-de-Haën (Sigma-Aldrich, L’Isle d’Abeau Chesnes, France), ¹³C-DEHP (1591 Bq/incubation) fortified when necessary with unlabelled DEHP were added in 5 μl ethanol to the culture medium in order to reach final concentrations of 0.5, 1, 2, 5, 10, 15 or 20 10⁻³ M of DEHP. All incubations were carried out in triplicate and lasted 24 h for TETAX (0.1 g tissue/well) or 48 h for CELIAS (1.10⁶ cells/well). At the end of the incubation, media were stored at −20°C. Explants were collected in 0.5 ml medium and homogenized using an Ultra-turrax disperser (IKA, Germany). Cells were collected in 0.5 ml trypsinized buffer before homogenization. The radioactivity in aliquots (50 μl) of each incubation medium and explant or cell homogenates was measured by counting using a Packard TriCarb 4430 counter with Ultima Gold (Perkin Elmer, France). High-performance liquid chromatography (HPLC, HP 1100) coupled to an online radioactivity detection system (Flo-One AS500), with Flo-scint II (Perkin Elmer, France) was used for metabolite profiling. The HPLC system consisted of a Kromasil C18 column (250 × 4 mm, 5 μm) coupled to a Kromasil C18 guard precolumn. Mobile phases consisted of acidified water (pH 2.8) and methanol (90:10 v/v) in A, and 100% methanol in B. The flow rate was 1 ml/min at 30°C; gradient: 0–35 min: linear gradient from 100% A to 100% B; 35–55 min: 100% B. Metabolites were identified by comparison of radio-HPLC retention time with those of authentic standards, except for MEHP whose structure was confirmed using liquid chromatography–mass spectrometry (MS/MS).
Measurement of testosterone production
Testosterone was assayed in duplicate using a specific radioimmunoassay (RIA; Immunotech, Beckman Coulter, Villepinte, France). The intra- and inter-assay coefficients of variation were 8.6 and 11.9%, respectively.

Measurement of insulin-like factor 3 production
Insulin-like factor 3 (INSL3) was assayed in duplicate using a commercial RIA (RK-035-27, Phoenix France, Strasbourg, France). The intra- and inter-assay coefficients of variation were ≤5.6% and 7.6%, respectively.

Measurement of inhibin B production
Inhibin B was measured using a commercial ELISA kit (DSL-10-84100 Active, Beckman Coulter, Villepinte, France). The intra- and inter-assay coefficients of variation were ≤5.6% and 7.6%, respectively.

Analysis of the pathways of androgen production
Solid phase extraction (SPE) C18 cartridges, reagents and solvents were of analytical or HPLC grade quality from Solvent Documentation Synthesis (SDS, Pepyin, France). Standard reference steroids were from Sigma (St Louis, MO, USA). Deuterated internal standards were from Steraloids (Wilton, NY, USA). Quantification was performed by isotopic dilution. Samples were spiked with 400 pg of internal standards (etiocholanolone-d5, 17α-testosterone-d3, dihydrotestosterone-d3, 19-androstenedione-d3, progesterone-d5, 17α-methyltestosterone-d3 and 17β-estradiol-d3). Samples were applied on a C18 SPE column (2 g stationary phase) previously conditioned with 10 ml methanol and 10 ml water. The column was washed with water (5 ml), followed by cyclohexane (5 ml), and steroids were eluted with methanol (10 ml). The extracts were dried (N2, 45°C) and 400 pg of external standard (norgestrel) was added. Derivatization procedure and measurements in gas chromatography (GC)–MS/MS in electronization mode were performed as already described (Prevost et al., 2004; Courant et al., 2008).

Statistical analysis
Values are means ± SEM, and expressed as percentages of the control value. They were analysed with the non-parametric signed rank Mann–Whitney test. A P-value inferior to 0.05 was considered statistically significant.

Results

Effect of the treatments on testis explant morphology
Exposure of the testicular explants to 10⁻⁵ M DEHP, MEHP did not reveal any alteration in their morphology. In contrast, ketoconazole caused an increase in the number of apoptotic germ cells (Fig. 1).

DEHP, MEHP and ketoconazole effects on human testosterone production
Incubation of testicular explants with 10⁻⁵ M ketoconazole resulted in an reduction in testosterone of 53% after 24 h of exposure and 59% after 48 h (Fig. 2A).

After 24 h of culture with 10⁻⁵ M DEHP, testosterone levels were reduced by 29%, whereas 10⁻⁴ M DEHP had no significant effect (Fig. 2A). Exposure to either 10⁻⁵ or 10⁻⁴ M MEHP resulted in a significant reduction in testosterone (Fig. 2A). Neither DEHP nor MEHP had any significant effect on testosterone after 48 h (Fig. 2A).

Distribution of DEHP and MEHP in the TEXAS
After 24 h, <3% of the 10⁻³ M of DEHP and <6% of the MEHP added to the cultures were found in the explants, and the rest was either found in the culture medium (41 and 94% for DEHP and MEHP, respectively), the PET insert (<5 and 1% for DEHP and MEHP, respectively), or bound to the plastic culture plate (51% for DEHP; Fig. 2B).

Biotransformation of DEHP
DEHP has to be metabolized into MEHP to be bioactive (Gray and Gangolli, 1986) and metabolites of MEHP, including SOH-MEHP (Koch et al., 2005), also display anti-androgenic properties (Chauvigne et al., 2009). A typical radio-HPLC chromatogram showing the bio-transformation of DEHP in the TEXAS is shown in Fig. 3. We found that DEHP was metabolized into MEHP, and that this MEHP was processed into SOH-MEHP. Qualitatively, similar results were obtained when using the CELIAS system. The use of seven different DEHP concentrations showed that the formation of MEHP and SOH-MEHP peaked at 7.8 ± 2.7 nmol/24 h/0.1 g tissue and 5.8 ± 0.78 nmol/24 h/0.1 g tissue, respectively, in the TEXAS, and at 24.66 ± 9.97 nmol/48 h/1.10⁶ cells and 9.97 ± 0.04 nmol/48 h/1.10⁶ cells, respectively, in the CELIAS.

Steroidogenic pathways of testosterone production
The global GC–MS/MS analysis of testosterone pathways confirmed that both ketoconazole and MEHP inhibited testosterone (−48 and −40%, respectively; Fig. 4). We also found that 4-androstene-3,17-dione, the precursor of testosterone, was significantly inhibited by ketoconazole (−47%) and by MEHP (−33%; Fig. 4A). Likewise, ketoconazole...
decreased the levels of 17OH-pregnenolone and DHEA. In contrast, the production of pregnenolone, which is the substrate of the CYP17,20 hydroxylase enzyme, was not decreased by ketoconazole (Fig. 4A). Production of all the precursors of testosterone of the Δ5 pathway was inhibited after exposure of the explants to MEHP (Fig. 4A). In the Δ4 pathway, ketoconazole decreased 17OH-progesterone and downstream testosterone precursor levels to a similar extent as testosterone (Fig. 4B). However, ketoconazole did not affect pregnenolone production, and actually caused a progesterone accumulation (+163%). Similar to the Δ5 pathway, MEHP significantly decreased all the tested steroids in the Δ4 pathway (Fig. 4B).

**DEHP, MEHP and ketoconazole and INSL3 production**

None of the treatments were found to affect Leydig cell INSL3 concentrations (Fig. 5).

**DEHP, MEHP and ketoconazole and inhibin B production**

In contrast to ketoconazole, which significantly increased inhibin B (+23%), neither DEHP nor MEHP had any effect on this Sertoli cell product (Fig. 6).
DEHP, MEHP and ketoconazole effects on testosterone in the CELIAS

DEHP, MEHP and ketoconazole all inhibited testosterone production by NCI-H295R cells in a dose-dependent manner (Fig. 7A). The effects of ketoconazole on testosterone decreased as the culture time was extended from 24 to 72 h (Fig. 7B). Both DEHP and MEHP inhibited testosterone after 24 h of exposure (−29 and −47%, respectively; Fig. 7B), and only a small difference was observed for DEHP between 24, 48 and 72 h (−32 and −22%, respectively; Fig. 7B); but the inhibitory action of MEHP declined between 48 and 72 h.

Discussion

This study is the first to provide direct evidence that phthalates have the ability to suppress human testicular steroidogenesis, and notably androgen synthesis. Previous studies have failed to provide evidence of any direct anti-androgenic effect of either MBP (Hallmark et al., 2007) or MEHP (Lambrot et al., 2009) in human fetal testis. This may be due to the differences between fetal and adult-type Leydig cells (Jackson et al., 1986; De Kretser and Kerr, 1988; Svechnikov et al., 2010), or to the differences in the culture conditions between the model systems. It cannot also be totally ruled out that the use
of human fetal testis explants to study the anti-androgenic action of phthalates is ‘limited’ as stated by Hallmark et al. (2007). It is noteworthy that the administration of dibutyl phthalate (DBP) to neonatal marmosets reduced serum testosterone concentrations (Hallmark et al., 2007).

We investigated whether DEHP and MEHP are metabolized in the human adult testis to (i) elucidate the significance of the anti-androgenic effects of DEHP, (ii) understand why the effects of DEHP and MEHP declined during the course of the cultures and (iii) help interpret the significance of our data to human exposure. We show that DEHP was processed into MEHP by the testis explants, and that the latter was further processed into SOH-MEHP. Therefore, the DEHP-induced inhibition of testosterone production by our models most probably results from the activities of MEHP and SOH-MEHP. The decline in the anti-androgenic effects observed after 48 h of culture most likely results from this biotransformation. Likewise, the time-dependent decrease in the anti-androgenic effects of both DEHP and MEHP in the CELIAS is also likely to be the result of biotransformation processes. In the CELIAS, more than 23% of the DEHP was found to be associated with the cells after 24 h of culture, with these cells being able to metabolize it. Treatment with $10^{-3}$ M DEHP inhibited testosterone, whereas $10^{-4}$ M DEHP had no effect after 24 h of culture. Under conditions in which DEHP is not metabolized into MEHP (i.e. in the fetal rat testis ex vivo), it exerts stimulatory effects on testosterone production (pro-androgenic effects; Chauvigne et al., 2009), so possibly when added at $10^{-4}$ M, the amount of DEHP in the culture before metabolization may be sufficient to counterbalance the anti-androgenic effects of MEHP and SOH-MEHP.

Using a method exploiting radio-labelled phthalates (Chauvigne et al., 2009), we were able to estimate that, when $10^{-3}$ M of the phthalates of interest were added for 24 h, about 3–6% of DEHP and MEHP were associated with the testis explants. Taking this into account along with our findings on the testicular DEHP biotransformation, we estimate that phthalate concentrations of the order of 1 μg/ml are able to display significant anti-androgenic effects. Interestingly, such concentrations of DEHP or MEHP have been found in the serum and semen of men (Buchta et al., 2005; Pan et al., 2006; Han et al., 2009; Meeker et al., 2009). Consistent with these observations, two epidemiological studies show associations between exposure to MEHP and lower free testosterone concentrations in men (Pan et al., 2006; Meeker et al., 2009).

Studies of the rat in vivo have shown that phthalate-induced testosterone suppression is mediated by the inhibition of a number of genes involved in cholesterol transport and metabolism, or of genes encoding steroidogenic enzymes (Shultz et al., 2001; Lehmann et al., 2004; Liu et al., 2005; Borch et al., 2006; Lahousse et al., 2006). The potential involvement of peroxisome proliferator-activated receptors has also been described (Maloney and Waxman, 1999; Bilty et al., 2004). In a recent study on the fetal rat testis ex vivo, we showed that the direct inhibitory effects of MEHP are mediated by the inhibition of the cyp17a1 gene and a decrease in the CYP17A1 protein (Chauvigne et al., 2011).

However, the results presented here show that all testosterone precursors, in both the Δ4 and Δ5 steroidogenic pathways, were significantly inhibited by exposure of the human testis to MEHP. As this study was not designed to investigate the mechanism of action of phthalates (or of ketoconazole) on human steroidogenesis, the precise mechanisms underlying the suppressing effects of MEHP remain to be elucidated.

Ketoconazole has been shown to lower serum testosterone in men (Pont et al., 1982; Schurmeyer and Nieschlag, 1982; Rajfer et al., 1986). Our results demonstrate strong inhibitory effect of ketoconazole on testosterone in the human testis and in the NCI-H295R cell line, as recently described (Hecker et al., 2006). Ketoconazole also increased germ cell apoptosis. It is likely that this ketoconazole-induced germ cell degeneration was at least in part, a consequence of the inhibition of testosterone, as androgen deprivation is known to result in a degeneration of germ cells (Steinberger, 1971).
Consistent with our findings, ketoconazole-treated rats display degeneration of germ cells (Amin, 2008). Interestingly, when the human testis samples were exposed to ketoconazole, pregnenolone was unaffected but progesterone accumulated, which confirms that the Cyp17 hydroxylase enzyme is a target of this drug (Albertson et al., 1988). Thus except for pregnenolone and progesterone, the concentrations of all the precursors of testosterone were found to be decreased by ketoconazole. This may also reflect ketoconazole-induced inhibition of cholesterol synthesis, which has previously been shown in vivo in men (Miettinen and Valtonen, 1984).

INSL3 is a Leydig cell product responsible for the first phase of testicular descent during fetal life and is believed to be involved in germ cell survival and bone metabolism (Ivell and Anand-Ivell, 2009; Bay and Andersson, 2011). Neither phthalates nor ketoconazole affected INSL3 production. This contrasts with evidence that prenatal exposure to DBP or MBP inhibits INSL3 gene expression in the rat fetal testis and may be associated with impaired testicular descent (Liu et al., 2005; Shono et al., 2005). It is also discordant with a report that the intensity of Leydig cell INSL3 immuno-labelling or the INSL3 gene expression was decreased in the fetal testes of most rats exposed in utero to phthalates (Wilson et al., 2004; McKinnell et al., 2005). These discrepancies are likely to reflect differences in the sensitivities of fetal and adult Leydig cells, as discussed above and in the approaches used.

We report that neither DEHP nor MEHP had an effect on inhibitor B, consistent with the maintained Sertoli cell integrity. Accordingly, in humans, studies have revealed that environmental exposure to DEHP is not associated with changes in inhibitor B serum levels in healthy men (Mendiola et al., 2010) or in men recruited through a study on infertility (Meeker et al., 2009). However, male rats exposed in utero and post-natally to DEHP show transitory abnormally low inhibitor B serum levels on post-natal Day 22 (Borch et al., 2004). Our observation that ketoconazole significantly increases inhibitor B levels is the first evidence that this compound can disrupt human Sertoli cell function. The reason why inhibitor B levels were found to be increased when germ cell apoptosis is rising after exposure to ketoconazole, while the reverse would be expected (De Kretser and Robertson, 1989), remains unknown.

The main conclusion of our study is that phthalate esters can alter human steroidogenesis. The active concentrations of phthalates which alter testosterone production appear to be within the range of concentrations found in men in recent epidemiological studies associating exposure to phthalates and impairment of androgenic status.

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Authors’ roles
B.J. and N.D.-R. designed the research; C.D.-L., O.A., E.P., F.C., and L.L. performed the research; B.L.B. and D.Z. designed the global analysis of the Δ5 and Δ4 pathways of androgen production and the bio-transformation experiments, respectively; and F.G. supervised and performed orchidectomies. All authors contributed to critically reviewing the draft manuscript. All authors saw and approved the final version of the report.

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Conflict of interest
The authors declare they have no competing interests be it financial, personal or professional.

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