Antagonistic Effects of Testosterone and the Endocrine Disruptor Mono-(2-Ethylhexyl) Phthalate on INSL3 Transcription in Leydig Cells

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Insulin-like 3 (INSL3) is a small peptide produced by testicular Leydig cells throughout embryonic and postnatal life and by theca and luteal cells of the adult ovary. During fetal life, INSL3 regulates testicular descent in males, whereas in adults, it acts as an antiapoptotic factor for germ cells in males and as a follicle selection and survival factor in females. Despite its considerable roles in the reproductive system, the mechanisms that regulate INSL3 expression remain poorly understood. There is accumulating evidence suggesting that androgens might regulate INSL3 expression in Leydig cells, but transcriptional data are still lacking. We now report that testosterone does increase INSL3 mRNA levels in a Leydig cell line and primary Leydig cells. We also show that testosterone activates the activity of the INSL3 promoter from different species. In addition, the testosterone-stimulating effects on INSL3 mRNA levels and promoter activity require the androgen receptor. We have mapped the testosterone-responsive element to the proximal INSL3 promoter region. This region, however, lacks a consensus androgen response element, suggesting an indirect mechanism of action. Finally, we show that mono-(2-ethylhexyl) phthalate, a widely distributed endocrine disruptor with antiandrogenic activity previously shown to inhibit INSL3 expression in vitro, represses INSL3 transcription, at least in part, by antagonizing testosterone/androgen receptor action. All together our data provide important new insights into the regulation of INSL3 transcription in Leydig cells and the mode of action of phthalates. (Endocrinology 149: 4688–4694, 2008)

INSLIN-LIKE 3 (INSL3), ALSO known as relaxin-like factor, is a member of the insulin-IGF-relaxin family of growth factors and hormones (1, 2). This hormone is almost exclusively expressed in the gonads. During fetal life, INSL3 is produced by maturing Leydig cells of the male gonad, whereas in adults it is secreted by both the testis (Leydig cells) and the ovary (theca and luteal cells) (1—4). Consistent with its sexually dimorphic expression pattern during fetal life, INSL3 was found to be a master regulator of gonadal descent, an essential step of the male sex differentiation process. INSL3−/− null mice have bilateral cryptorchid testes located high in the abdominal cavity close to the kidneys (5, 6). Further support for INSL3 as a regulator of testicular descent came from elegant transgenic experiments in which INSL3 was overexpressed in pancreatic β-cells of INSL3 null mice (7). In these animals, normal testis descent was restored. Even more convincing was the generation of female transgenic mice overexpressing INSL3, which led to descended ovaries (7, 8). In adult rats, INSL3 was found to be a master regulator of gonadal descent, an essential step of the male sex differentiation process. INSL3−/− null mice have bilateral cryptorchid testes located high in the abdominal cavity close to the kidneys (5, 6). Further support for INSL3 as a regulator of testicular descent came from elegant transgenic experiments in which INSL3 was overexpressed in pancreatic β-cells of INSL3 null mice (7). In these animals, normal testis descent was restored. Even more convincing was the generation of female transgenic mice overexpressing INSL3, which led to descended ovaries (7, 8). In adults, INSL3 acts as a germ cell survival factor (9) and would also be involved in follicle selection and survival (10, 11).

Despite its important roles in reproductive development and function, surprisingly very little is known regarding the mechanisms regulating INSL3 expression in gonadal cells. At the molecular level, so far only two transcription factors have been shown to regulate INSL3 promoter activity in Leydig cells: the nuclear receptors steriodogenic factor-1 (SF1; Ad4BP/NR5A1) and NUR77 (NGFI-B/NR4A1) (12—16). At the hormonal level, INSL3 expression was found to require the pituitary LH in postnatal Leydig cells (3, 9, 17). This, however, is believed to be associated with the differentiation status of Leydig cells, which is dependent on LH, rather than the result of an acute response to LH as for steroidogenesis (3, 18). In addition to LH, there is mounting evidence that INSL3 expression would be regulated by androgens in several species. In testicular feminized mice, which harbor a mutation in the androgen receptor (AR) gene causing complete androgen insensitivity (19—21), INSL3 expression is severely decreased (22). In an AR hypomorphic mice model in which AR levels are reduced (23), INSL3 expression was down-regulated nearly 10-fold (24). In these mice, LH and testosterone levels were dramatically increased, consistent with normally differentiated and functioning pituitary gonadotrope and testicular Leydig cells (24). In adult rats, GnRH antagonists (chemical castration) down-regulate INSL3 expression (9). Treatment with human chorionic gonadotropin restored INSL3 mRNA levels in GnRH antagonist-treated animals (9) and hpg mice (3), which have a deletion of the Gnrh1 gene (25, 26). In humans, INSL3 and testosterone concentrations are significantly correlated (17, 18). Finally, endocrine disruptors exhibiting antiandrogenic activity, such as the widely distributed plasticizer phthalate, repress INSL3 expression in Leydig cells (27—29). The exact mechanisms of action of an-
drogens on Insl3 expression, however, remain completely obscure.

In the present study, we provide evidence that testosterone regulates Insl3 transcription in Leydig cells in an AR-dependent manner. We have also mapped an androgen-responsive region in the human INSL3 promoter. Finally, we found that mono-(2-ethylhexyl) phthalate (MEHP) represses Insl3 transcription, at least in part, by antagonizing the testosterone stimulating effects.

**Materials and Methods**

The −1137−, −920, and −656 to +11 bp human INSL3 promoter fragment have been previously described (15). Additional deletion constructs to −132 and −85 bp were generated by PCR using the −1137 bp construct as template along with a common reverse primer containing a KpnI cloning site (underlined) 5'-GGG GTA CCG GTG GTG GCC GGC AAG C-3' and the following forward primers that contain BamHI cloning sites (underlined): −132 bp, 5'-GGG GAT CCC AGA AAG GCT CTG GAC C-3' and −85 bp, 5'-GGG GAT CCC GCC CTA AAG GAC CCC TGG GAG AAA TTA TCT GGC ACT AAC CCC ACC C-3' and antisense 5'-GAT CCG CCT ATA AAG GGG GAC CCC GCT TGG GCC CGC CAC CCA CCG GTA C-3'.

Various trinucleotide mutant constructs in the context of the −1137− and −132 bp reporters were generated using the QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA) along with the following oligonucleotides (only the sense oligonucleotide is shown) in which the nucleotides (underlined) and antisense 5'-GGG GTA CCG GTG GGT GGC GCC GGC AAG C-3' and +11 bp

**Fig. 1.** Testosterone stimulates Insl3 expression in Leydig cells. A, MA-10 Leydig cells were treated with either 1500 nM dimethylsulfoxide (open bar) or increasing doses of testosterone (T) (black triangle: 0.15, 1.5, 15, 150, and 1500 nM) for 36 h. B, MA-10 and primary cultures of Leydig cells from rats were treated with 15 nM dimethylsulfoxide (open bars) or testosterone (T) (black bars) for 36 h. Total RNA was next isolated and used in quantitative real-time PCR using primers specific for Insl3 cDNA as described in Materials and Methods. Results were corrected with the Rpl19 cDNA. Results are the mean of three individual experiments performed in duplicate (±SEM).

**Fig. 2.** The INSL3 promoter from different species is activated by testosterone. A, MA-10 cells were transfected with a −1137 bp human INSL3 promoter construct and treated with either 1500 nM dimethylsulfoxide (open bar) or increasing doses of testosterone (T) (black triangle: 0.15, 1.5, 15, 150, and 1500 nM) for 36 h. A different letter indicates a statistically significant difference. B, MA-10 cells were transfected with a −1137 to +5 bp mouse INSL3 promoter, −978 to +5 bp mouse Insl3 promoter and −508 to +5 bp rat Insl3 promoter and treated with 15 nM dimethylsulfoxide (open bars) or testosterone (T) (black bars) for 36 h. *, Statistically significant difference from the respective control. Results are shown as fold activation over control (±SEM).
FIG. 3. Testosterone-dependent activation of Insl3 transcription requires the androgen receptor. A, MA-10 Leydig cells were transfected with a −1137-bp human Insl3 promoter construct and pre-treated with 50 μM dimethylsulfoxide (open bars) or increasing doses of hydroxyflutamide (HF) (black triangle: 50 nM, 250 nM, 500 nM, 5 μM, 25 μM, and 50 μM) for 1 h followed by addition of vehicle (−) or 15 nM testosterone (T, +) for 36 h. **statistically significant difference from the respective control. Results are shown as fold activation over control (SEM). B, MA-10 cells were treated with either dimethylsulfoxide (open bar), 15 nM testosterone (T) (black bar), 5 μM hydroxyflutamide (HF) (gray bar), or a combination of hydroxyflutamide and testosterone (hatched bar). Total RNA was isolated and Insl3 expression was determined by quantitative real-time PCR. Results were corrected with the Rpl19 cDNA. Results are the mean of three individual experiments performed in duplicate (+SEM). *, statistically significant difference from control.

Cell culture and transfections

The mouse Leydig cell line MA-10 (31) was provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). Cells were grown in Waymouth’s media supplemented with 12% horse serum and 50 μg/liter of gentamicin and streptomycin sulfate, at 37°C in 5% CO2. Cells were plated in a 24-well plate at a density of 90,000 cells/well in Waymouth’s supplemented with 12% charcoal-treated horse serum and allowed to recover for 16–24 h before transfection. An even stronger response was observed when primary Leydig cell cultures were used (Fig. 1B). These results indicate that Insl3 expression is regulated by androgens in Leydig cells and that the MA-10 Leydig cell line constitutes an appropriate model to study the underlying mechanisms of androgen action.

Isolation of primary Leydig cells

Primary Leydig cells were isolated as described previously (32) from 35-d-old Sprague Dawley rats obtained on site. Serum-free medium 199 with Earle’s salts (Sigma-Aldrich Canada, Oakville, Ontario, Canada), t-glutamine, 1.5 mM HEPES, 2.5 g/liter NaHCO3, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) was used during preparation and culturing. After 2 d in culture, the purity of the Leydig cell preparation was evaluated to be about 95% enriched as assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase activity (33). All experiments were conducted according to the Canadian Council for Animal Care and have been approved by the Animal Care and Ethics Committee of Laval University (protocol 2005-184).

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated using the RNeasy Plus minikit (QIAGEN Canada, Mississauga, Ontario, Canada). First-strand cDNAs were synthesized from a 5-μg aliquot of the various RNAs using the Superscript III reverse transcriptase system (Invitrogen Canada). Quantitative real-time PCR for Insl3 and Rpl19 were performed as previously described (15, 34). Each amplification was performed in duplicate using at least three different preparations of cDNAs for each of the three different RNA extractions.

Statistical analysis

Statistical analyses were done using one-way ANOVA or ANOVA on ranks (when parameters were not met) followed by a Student-Newman-Keuls posttest. For all statistical analyses, P < 0.05 was considered significant. All statistical analyses were done using the SigmaStat software package (Systat Software Inc., San Jose, CA).

Results

Testosterone increases Insl3 mRNA levels in Leydig cells

To assess whether androgens could regulate Insl3 expression, we first treated MA-10 cells, a Leydig cell line known to express AR (35), with increasing doses of testosterone, doses that are within the physiological range found within the rodent testis (200–350 nm) (36, 37). As shown in Fig. 1A, Insl3 mRNA levels were significantly increased in response to testosterone as determined by quantitative real-time PCR. An even stronger response was observed when primary Leydig cell cultures were used (Fig. 1B). These results indicate that Insl3 expression is regulated by androgens in Leydig cells and that the MA-10 Leydig cell line constitutes an appropriate model to study the underlying mechanisms of androgen action.

FIG. 4. The testosterone-responsive area is located in the proximal Insl3 promoter. MA-10 cells were transfected with various 5′ deletion constructs of the human Insl3 promoter (the 5′ end point of each construct is indicated on the left of the graph) and treated with dimethylsulfoxide (open bars) or 15 nM testosterone (T) (black bars). *, statistically significant difference from the respective control.
Testosterone stimulates Insl3 promoter activity from different species

Next, we ought to determine whether the effects of testosterone could be due to an increase in Insl3 gene transcription. As shown in Fig. 2A, treatment of MA-10 Leydig cells with increasing doses of testosterone led to a dose-dependent activation of a −1137-bp human INSL3 reporter construct. Similar results were obtained with the rat and mouse Insl3 promoters (Fig. 2B). Thus, testosterone can activate the Insl3 promoter from different species.

Testosterone acts in an AR-dependent manner to increase in Insl3 transcription

Although testosterone is known to act mainly through AR, some AR-independent responses to androgens have been reported (reviewed in Ref. 38). The involvement of AR in testosterone-induced Insl3 transcription was assessed by treating MA-10 Leydig cells with testosterone in the presence or absence of hydroxyflutamide, the biologically active form of flutamide and a potent AR antagonist. Testosterone-mediated increase in INSL3 promoter activity (Fig. 3A) and endogenous mRNA levels (Fig. 3B) were abolished in the presence of the antagonist. The AR antagonist had no effect when used alone (Fig. 3). All together, these results establish the requirement of AR for testosterone-dependent stimulation of Insl3 transcription.

Mapping of the testosterone-responsive element in the INSL3 promoter

To locate the testosterone-responsive element in the human INSL3 promoter, a series of 5' progressive deletion constructs were generated and transfected in MA-10 Leydig cells that were then treated with 15 nM testosterone. As shown in Fig. 4, deletion from −1137 to −132 bp had no effect on testosterone responsiveness (nearly 3-fold). Further deletion to −85 bp severely blunted the testosterone-responsiveness of the INSL3 promoter to 1.4-fold. The testosterone-activating effects were specific because a −31-bp minimal INSL3 promoter construct was no longer responsive (Fig. 4). These data indicate that the main testosterone-responsive element(s) are located between −132 and −85 bp. A survey of the 47-bp promoter sequence between −132 and −85 bp did not reveal the presence of a consensus androgen response element (GGTACAnnnTGTTCT). The 47-bp region, however, contains a half-site for the binding of nuclear receptors (AGGTCA), which we previously described as a binding site for the nuclear receptors NUR77 and SF1 (15, 16). To better define the element within the 47-bp region, a series of mutant constructs were generated in the context of the −1137-bp (Fig. 5A) and the −132-bp (Fig. 5B) INSL3 promoter. In the context of the −1137-bp promoter construct, mutations M1-M3 had no impact on the testosterone-responsiveness of the INSL3 promoter, whereas mutations M4-M7 caused a
small but significant reduction in the testosterone-dependent activation (Fig. 5A). Mutations M8, M9, and M10, sequentially changing each of the underlined triplet in the sequence GACCTTTGCC, had the most dramatic effects, decreasing testosterone responsiveness to the level observed with the −85-bp construct (from nearly 3-fold to 1.4-fold) (Fig. 5A). Interestingly, this sequence contains the NUR77/SF1 nuclear receptor binding site. Similar results were obtained in the context of the −132-bp reporter construct with mutation M9 eliminating the testosterone-responsiveness of the INSL3 promoter (Fig. 5B).

**MEHP antagonizes testosterone-induced Insl3 expression**

MEHP, the active metabolite of a widely used plasticizer, is known to repress Insl3 expression in vivo in Leydig cells (29). Although the exact molecular mechanisms remain unidentified, it is known that MEHP cannot bind to AR but nonetheless acts as an antiandrogen (39–41). Because we have shown that androgens activate Insl3 transcription, we tested whether MEHP could modulate testosterone action on Insl3 transcription. As shown in Fig. 6, 30 μM of MEHP significantly inhibited the testosterone-mediated increase in Insl3 mRNA in primary Leydig cells but had no effect in unstimulated cells. Similarly, MEHP, but not its inactive precursor DEHP, significantly reduced the testosterone-dependent activation of the human INSL3 promoter (Fig. 7). Thus, MEHP represses Insl3 expression, at least in part, by antagonizing testosterone action.

**Discussion**

INSL3, a small peptide of gonadal origin, plays important roles in reproductive function (42). In addition, its expression in males is highly correlated with the functional status of testicular Leydig cells and to circulating testosterone levels (17, 18, 43–45). In the present study, we found that androgens activate Insl3 transcription in Leydig cells and that this is partly antagonized by MEHP, a widely distributed endocrine disruptor with antiandrogenic activity.

**Expression of Insl3 in Leydig cells**

Although the Insl3 gene is located within the last intron of the Jak3 gene, the two genes have very divergent expression profiles. Jak3 is restricted to lymphoid and hematopoietic cells (46, 47), whereas Insl3 is found exclusively in the gonads, mainly in testicular Leydig cells (1–4). Jak3 and Insl3 are therefore not likely to share regulatory elements despite their close genomic association. Because of this atypical organization, it is believed that the regulatory motifs driving Insl3 expression in Leydig cells are located within a relatively short 5’ flanking sequence. In agreement with this, the first 200 bp upstream of the transcription start site were found to be sufficient to confer activity to the mouse (12, 13), rat (14), and human (our data) Insl3 promoter in MA-10 Leydig cells. Within this fragment are found binding sites for the transcription factors SF1 and NUR77 (12–16). Consistent with an in vivo role for SF1, Insl3 expression was reduced in a Leydig cell-specific Sf1 knockout mouse model (48).

**Mechanism of androgen action on Insl3 transcription**

In addition to these two transcription factors, we found that testosterone also activates Insl3 expression in a mouse Leydig cell line and primary Leydig cells from rats. These data are supported by evidence from the literature whereby modulation of androgen production or action in animal models was shown to cause a concomitant alteration in Insl3 mRNA levels (3, 9, 17, 18, 22, 24, 49).

We found that testosterone directly regulates the activity of the Insl3 promoter from various species. This indicates that
the mechanism of androgen action on Insl3 expression has been evolutionarily conserved despite the poor sequence conservation between the human and rodent Insl3 promoter. Testosterone action on Insl3 transcription was found to be AR dependent. The AR-responsive region that we mapped in the human Insl3 promoter, however, does not contain an androgen response element, suggesting that androgen-activated AR acts in a DNA-binding-independent manner. AR has been shown to modulate gene expression and cell function without directly binding to DNA through nonclassical pathways (reviewed in Refs. 50 and 51).

Androgen-MEHP antagonism in Insl3 transcription

Phthalates are chemicals used to impart flexibility and durability to polyvinyl chloride and comprise up to 40% of the plastic volume. Phthalates are ubiquitous contaminants of the environment and humans, and animals are inevitably exposed to these chemicals (52–58). The primary targets of phthalates are testicular Leydig cells (59).

In the present study, we found that MEHP, a biologically active phthalate with endocrine disrupting activity, partly inhibited testosterone action on Insl3 expression in a Leydig cell line (MA-10) and primary cultures of rat Leydig cells. Our data confirm that MA-10 cells are an appropriate model to study phthalate-mediated effects, which is in agreement with a previous study by Dees et al. (60). It is known that in utero exposure to phthalates, including MEHP, causes several detrimental reproductive defects, including undescended testis, hypospadias, and decreased testosterone production, in males in various animal models (reviewed in Ref. 61). They are believed to have adverse effects on human reproductive health as well (reviewed in Ref. 62). Phthalate exposure results in decreased expression of Insl3 and genes involved in testosterone biosynthesis in Leydig cells (reviewed in Refs. 63 and 64). Although phthalates have antiandrogenic effects, they cannot interact with AR (39–41). Phthalate repressive effects in Leydig cells are believed to be mediated, at least in part, through activation of the peroxisome proliferator-activated receptor (PPAR) (65, 66). In the testis of PPARα-deficient mice exposed to phthalates, expression of the peripheral-type benzodiazepine receptor, which is involved in cholesterol transport, was no longer repressed (67), and the overall testis structure was found to be predominantly normal (68). Activated PPAR can repress gene expression in a DNA-binding-independent manner through the recruitment of corepressors or by interfering with other DNA-associated transcription factors (65, 66). Recently phthalates were also reported to act by decreasing recruitment of the transcription factor CCAAT/enhancer-binding protein-β to DNA but having no effect on SFI (69). Although the exact mechanism remains to be identified, we found that the phthalate MEHP antagonizes AR action through a region of the Insl3 promoter known to bind transcriptional activators, which suggests that disruption of transcription factor interactions might be involved.

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