

Involvement of Insulin-Like Factor 3 (Insl3) in Diethylstilbestrol-Induced Cryptorchidism

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Abstract

Recently, it has been shown that targeted inactivation of the *Insl3* gene in male mice results in cryptorchidism. The *Insl3* gene encodes insulin-like factor 3 (Insl3), which is expressed in fetal Leydig cells. The testicular factor Insl3 appears to play an important role in the transabdominal phase of testis descent, which involves development of the gubernaculum. Other studies have demonstrated that *in utero* exposure to diethylstilbestrol (DES), a synthetic estrogen, can lead to cryptorchidism both in humans and in animal models.

The present study was undertaken to investigate whether prenatal DES-exposure might interfere with testicular Insl3 mRNA expression. Furthermore, the effect of DES on steroidogenic factor 1 (SF-1) mRNA expression level was determined, since it has been shown that SF-1 plays an essential role in transcriptional activation of the *Insl3* gene promoter. Timed pregnant mice were treated with DES (100 µg/kg body weight) or vehicle alone on days E9 (gestational day 9) through E17. Control and DES-exposed mouse fetuses were collected at E16, E17 and E18, when transabdominal testis descent is taking place. Lack of gubernaculum development in DES-exposed animals was confirmed by histological analyses at E17. Expression of Insl3 and SF-1 mRNAs was studied in testes of control and DES-exposed fetuses at E16 and E18 by RNase protection assay. Prenatal DES-exposure resulted in a three-fold decrease in Insl3 mRNA expression level ($P < 0.005$), at both E16 and E18. In contrast, DES treatment had no effect on the expression of SF-1 mRNA. These results support our hypothesis that DES may interfere with gubernaculum development by altering Insl3 mRNA expression, providing a possible mechanism by which DES may cause cryptorchidism.

Diethylstilbestrol (DES) is a non-steroidal synthetic estrogen, which has been used as estrogen therapy to prevent abortion, mainly in the 1950s and 1960s (1). The usage of DES was banned in 1971, when it was found that *in utero* exposure to diethylstilbestrol (DES) is associated with urogenital tract abnormalities in female and male offspring. One of the abnormalities found in DES-exposed male offspring was cryptorchidism.

A biphasic model for the hormonal regulation of testis descent has been proposed (2). During the first or transabdominal phase, the testis moves from its initial position near the kidney to the abdominal bottom. In mice, this phase takes place between embryonic days E15.5 and E17.5 (3). The transabdominal movement of the testis is dependent on the differential development of two ligaments, the gubernaculum, which connects the caudal pole of the testis to the bottom of the abdomen and the cranial suspensory ligament (CSL) which is running between the gonad and the diaphragm (4,5). During the transabdominal phase, the male gubernaculum is growing and differentiating whereas outgrowth of the CSL is lacking. It has been shown in animal models that exposure to exogenous estrogens, including DES, disrupts the first phase of testis descent,

leading to maldescent (3,6,7). It has been suggested that the cryptorchidism induced by DES is due to failure of gubernaculum development (6,7). The mechanism by which estrogens inhibit gubernaculum development is poorly understood.

Insulin-like factor 3 (Insl3), also designated Leydig insulin-like factor (Ley I-L) or relaxin-like factor (RLF), belongs to the insulin-like hormone family (8,9). The *Insl3* gene is specifically expressed in pre- and postnatal Leydig cells of the testis and in postnatal theca cells of the ovary (10). Recently, *Insl3* knockout mice, homozygous for a targeted inactivation of the *Insl3* gene (*Insl3*^{-/-} mice), have been generated (11,12). As a conspicuous aspect of the phenotype, male *Insl3*^{-/-} mice show disturbed testis descent resulting in cryptorchidism. Histological analysis of male *Insl3*^{-/-} fetuses revealed that the development of the gubernaculum is severely affected. This indicates that Insl3 might be an important factor for gubernaculum development in the mouse, during the transabdominal phase of testis descent. The position of the gonads in neonatal male *Insl3*^{-/-} mice (11,12) is strikingly similar to that of neonatal male mice prenatally exposed to DES (7); the testes are located high in the abdomen, near the lower poles of the kidneys and gubernaculum development is lacking. This led us to suggest that Insl3 expression might be impaired in testes of DES-exposed male fetuses, thereby providing a mechanism for DES-induced testis maldescent.

Steroidogenic factor-1 (SF-1) is an orphan nuclear receptor which is essential for gonadal development and sex

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differentiation (13). Both Sertoli and Leydig cells in the fetal testis express SF-1 (14). It has been shown that SF-1 plays an essential role in transcriptional activation of a number of genes, including an action on the *Insl3* gene promoter (15). Thus, altered *Insl3* gene expression might be a consequence of a changed level of SF-1.

We studied the effects of DES on transabdominal testis descent via histological analysis and determined whether DES alters *Insl3* and SF-1 mRNA expression during this phase by RNase protection assay.

Materials and Methods

Animals, treatment and collection of tissue

Adult mice (FVB strain) were housed under standard animal housing conditions in accordance with NIH Guide for the Care and Use of Laboratory Animals. To obtain timed pregnancies, female mice were placed in individual cages with male mice and the morning a vaginal plug was found was designated day 0 (E0) of pregnancy. Pregnant mice were injected subcutaneously on days E9 through E17, with diethylstilbestrol (DES; 100 $\mu\text{g}/\text{kg}$ body weight; Janssen Chimica, Beerse, Belgium) in olive oil or with olive oil alone (controls). Pregnant mice were sacrificed on E16, E17 or E18 by cervical dislocation and fetuses were quickly removed. E16 and E18 fetuses were examined under a dissection microscope, and after establishing the position of the testes, these were isolated, frozen in liquid nitrogen and stored until RNA isolation. E17 fetuses were used for histological analysis. These were fixed in 10% formalin and embedded in paraffin. Serial sections of 7 μm were cut and stained with hematoxylin-eosin.

RNA analysis

Total tissue RNA was isolated from pooled testes of 3 control or 3 DES-exposed fetuses using a modified guanidine thiocyanate procedure (16). For ribonuclease (RNase) protection assay (17), antisense cRNA probes were prepared by *in vitro* transcription using T7 RNA polymerase and [^{32}P]UTP (400 Ci/mmol; Amersham, Buckinghamshire, United Kingdom). A mouse *Insl3* cDNA fragment (10), a mouse SF-1 cDNA fragment (18) and a rat glyceraldehyde 3-phosphate dehydrogenase (GAPD) cDNA fragment were used as templates. An amount of 10 μg total RNA was hybridized with the purified labeled probes of GAPD and *Insl3* or SF-1 (5×10^4 cpm each) for 16 h at 55°C. After hybridization, samples were treated with RNase-A/T1 mixture for 1 h at 30°C and phenol/chloroform extracted. After precipitation, samples were resuspended in formamide loading buffer, and run on acrylamide-urea denaturing gels. Quantification of protected RNA fragments was performed using PhosphorImage analysis (Molecular Dynamics, B & L systems, Zoetermeer, The Netherlands). The mRNA levels of *Insl3* and SF-1 were normalized to GAPD to correct for differences in the amounts of RNA that were hybridized with the probes.

Statistics

Statistical analysis was performed by Student's two-tailed *t* test.

Results

Morphology

Male fetuses at E16 and E17 were examined under a dissection microscope to study the position of the testes, followed by dissection of the testes for RNA analysis. In control male fetuses at E16, the transabdominal descent of the testis was taking place; the testes were located well below the kidneys, just above the top of the bladder. However, in all DES-treated males at E16, the testes were found at a relatively high abdominal position, and in the majority of these animals (11/15), the testes were still located at the lower pole of the kidneys. At E18, the testes of control fetuses were located next to the bladder, at the bottom of the abdomen. In DES-treated male fetuses at the same fetal age, the testes were always positioned higher in the abdomen when compared to the controls, ranging from firm attachment to the posterior pole of the kidneys to a location well above the bladder.

Histological analysis of control and DES-treated fetuses at E17 showed an undifferentiated, female-like gubernaculum in DES-treated animals (Fig. 1). The Müllerian ducts had almost completely regressed in the control fetuses, but were still present in the DES-exposed male fetuses (not shown).

RNase protection assay

Expression of *Insl3* and SF-1 mRNAs in testes of control and DES-exposed fetuses at E16 and E18 was examined by RNase protection assay (Fig. 2). PhosphorImage analysis of a representative RNase protection assay for *Insl3* and GAPD

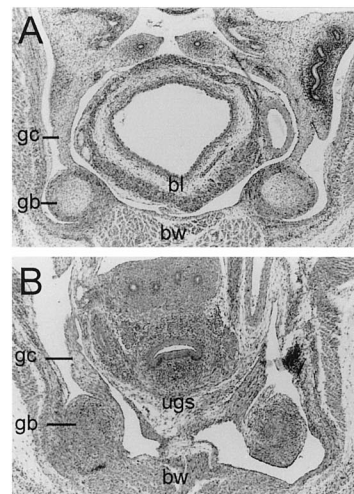


Fig 1. Inhibition of gubernaculum development by DES. Morphology of the lower abdomen of control and DES-treated fetuses at E17. In the control male fetus (A), the gubernaculum bulb is well-developed, with an inner mesenchymal core and muscular outerlayer. In contrast, in the DES-treated male fetus (B) the gubernaculum appears to be undifferentiated. bw, body wall; bl, bladder; gb, gubernaculum bulb; gc, gubernaculum cord; ugs, urogenital sinus.

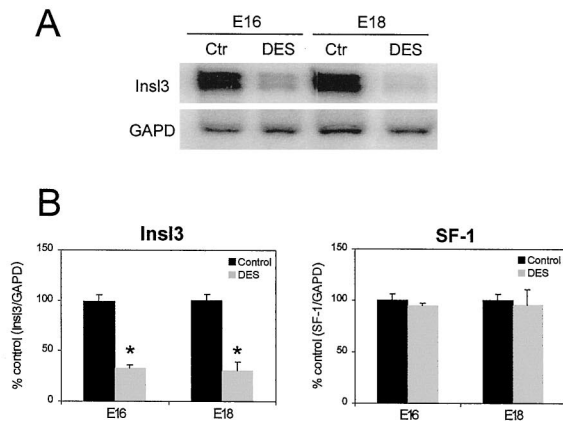


Fig 2. Effect of DES on testicular expression of *Insl3* and SF-1 mRNAs.

(A) PhosphorImage analysis of a representative RNase protection assay for *Insl3* and GAPD mRNA expression at E16 and E18. GAPD mRNA analysis was included as a control for RNA loading. (B) Quantification of *Insl3* and SF-1 mRNA levels in DES-exposed fetuses at E16 and E18. Levels of *Insl3* and SF-1 mRNA were normalized to that of GAPD mRNA. Values are expressed as percentage of control (considered 100% at each time point) and are the mean \pm SEM of at least two independent experiments performed in duplicate. *, $P < 0.005$ vs. control.

mRNAs is shown in Fig. 2A. Quantitative analysis revealed that, at both E16 and E18, *Insl3* mRNA expression in testes of DES-exposed fetuses was decreased by 70% compared with controls, as illustrated in Fig. 2B. In testes from control fetuses at E16, *Insl3* mRNA expression was similar to the expression level of this mRNA at E18. With regard to the possible involvement of SF-1 in *Insl3* expression, the present results show that SF-1 mRNA expression in testis of DES-treated fetuses was not significantly different from its expression in testis of control fetuses, at both E16 and E18.

Discussion

In the present study, prenatal exposure of male mouse fetuses to DES inhibits gubernaculum development during the transabdominal phase of testis descent, which is in agreement with published data (7). To date, the mechanism by which estrogens inhibit gubernaculum development has remained unclear. Hadziselimovic *et al.* (3) demonstrated that estrogens inhibit Leydig cell development and consequently the secretion of testosterone. It was proposed that estrogens suppress the function of the fetal pituitary gland. However, fetal development of Leydig cell function is independent of pituitary function (19, 20). The observation that the testis of hypogonadal (*hpg*) mice, lacking gonadotropin-releasing hormone, descend to the abdominal bottom with normal gubernaculum development, excludes a role of the fetal pituitary gland in transabdominal testis descent (21). Moreover, the role of estrogens in the ontogeny of undescended testes also appears to be independent of their effects on androgen action (22). Although mice with

complete androgen resistance (testicular feminization, *Tfm*) are cryptorchid, the transabdominal phase of testis descent is not affected in these mice (23). Exposure of *Tfm* mice to estrogen inhibits the development of the gubernaculum (22). In addition, exposure of wild-type fetal mice to estrogen in combination with testosterone does not reverse the estrogen-induced cryptorchidism (24).

Hutson and Donahoe (25) have suggested a possible role for anti-Müllerian hormone (AMH) in regulation of the first phase of testis descent, although direct evidence for such a role is lacking. It has been proposed that estrogens might interfere with AMH action. However, studies from Majdic *et al.* (14) and Visser *et al.* (26) could not demonstrate an effect of DES on AMH mRNA expression in fetal testicular tissue around the time of transabdominal descent.

In the present study, prenatal exposure to DES from E9 through E17 decreased the expression of *Insl3* mRNA in mouse testis, both at E16 and E18. Recently developed *Insl3* knockout mouse models demonstrated the requirement of *Insl3* in the transabdominal descent of the testis (11,12). It was proposed that *Insl3* is a third testicular factor, next to androgens and AMH, which is involved in male sex differentiation. *Insl3* is clearly involved in gubernaculum development, either directly or indirectly. Whether or not *Insl3* acts directly on the gubernaculum, reduced expression of *Insl3* mRNA in fetal testes by exposure to exogenous estrogens might be the mechanism by which estrogens inhibit gubernaculum development and cause testis maldescent. Under the present experimental conditions, prenatal exposure to DES did not affect SF-1 mRNA expression in mouse testis, measured at E16 and E18. Based upon a previous study done by Visser *et al.* (26), an effect of DES on the general development of DES-exposed fetuses can probably be excluded as a possible explanation for the decrease of *Insl3* mRNA. This is strengthened by the present observation that SF-1 mRNA expression is not affected. In contrast, Majdic *et al.* (14) demonstrated a decrease in SF-1 mRNA in testis of DES-exposed rat fetuses. However, the latter result is based on the use of rats and a different DES dose and treatment schedule.

It remains to be determined whether DES directly represses transcriptional activity of the *Insl3* gene in fetal Leydig cells, or causes nonspecific dysfunction of fetal Leydig cells which indirectly leads to impaired *Insl3* production. Furthermore, a direct action of DES upon the gubernaculum cannot be excluded.

Cryptorchidism can be experimentally induced in animals by prenatal exposure to DES, but also occurs in sons whose mothers have been given DES during pregnancy (1). In humans, the *INSL3* gene has been identified and characterized (27), and *in situ* hybridization on human testis sections demonstrated that *Insl3* is exclusively expressed in Leydig cells (28). It is of extreme interest to obtain information on the role of *INSL3* in testis descent in the human and such a study could lead to direct analysis of the effect of DES on *INSL3* expression in human males. This would not only give insight into the role of DES in the etiology of cryptorchidism, but would also be useful in view of the present concerns about the possible effects of

environmental estrogens on fetal development of the reproductive system (29).

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