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Insulin-like growth factor I (IGF-I) regulates endocrine activity of the embryonic testis in the mouse

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Abstract

Insulin-like growth factor I (IGF-I) is important for gonadal and reproductive functions in mammals, although the physiological role of this growth factor during gonadal development in rodents remains largely unknown. Here, we examined the steady-state levels of IGF-I mRNA by the reverse transcriptase polymerase chain reaction (RT-PCR). IGF-I protein expression was also detected by Western blot. The effect of IGF-I as promoter of 17α-hydroxylase/C17–20 lyase and 17β-hydroxysteroid dehydrogenase enzyme activity in vitro was evaluated by radioimmunoassay. Onset of IGF-I gene expression was on day E10 (urogenital ridge stage). IGF-I mRNA expression was markedly reduced on days E12 and E13 (testicular differentiation stage). IGF-I transcripts increased on day E14 and their transcription levels were maintained throughout the stages analyzed. Several IGF-I protein bands of 31–100 kDa were observed. Culture experiments demonstrated that 17α-hydroxyprogesterone and testosterone (T) secretion levels increased in the presence of IGF-I on days E11–E17. Additive effects of IGF-I plus $(Bu)_2$cAMP were also seen during testicular development. It is proposed that IGF-I regulates the expression of key steroidogenic enzymes important for endocrine activity of the testis during prenatal development leading to establishment of the male phenotype and fertility.

Keywords: IGF-I; Testis; Growth factors; 17α-Hydroxylase/C17–20 lyase; 17β-Ketosteroid reductase; 17β-Hydroxysteroid dehydrogenase; Testosterone; Leydig cells

1. Introduction

Testicular function is regulated by the gonadotrophins LH and FSH during postnatal development [1,2]. Recently, accumulating evidence has indicated that locally produced growth factors, among them insulin-like growth factor I (IGF-I) regulate testicular function [3,4]. It has been hypothesized that this growth factor acts as an autocrine/paracrine regulator of Leydig cell function [3,5,6]; however, the dual role of IGF-I remains to be established. Previous studies demonstrated IGF-I gene expression in the adult rat [7] and in fetal rat testis [8,9]. Moreover, it has been reported that several IGF-I transcript species are produced within rat fetal testis [8]. IGF-I mRNA was observed in the interstitial compartment of the mouse testis at 14 days postpartum (d.p.p.), whereas IGF-I transcripts were located in the spermatids at 30 d.p.p. [10]. IGF-I immunoreactivity has been found in human [11], rat [12], and mouse testes [13]. IGF-I protein is mainly detected in the spermatogonium 2 weeks after birth, and only pachytene spermatocytes and spermatids express it at 30 d.p.p. [14,15]. Culture experiments have demonstrated the presence of IGF-I in peritubular, Sertoli and Leydig cells in the rat [13,14]. More recently, it was reported that fetal rat testes secrete higher amounts of IGF-I than those contained in the embryonic male gonad [8]. Cultured adult rat Sertoli cells and immature porcine Leydig cells enhance IGF-I secretion in the presence of FSH and LH, respectively [15–17]. Moreover, IGF-I increases postnatal mRNA of key steroidogenic enzymes and their protein activity [18–22]. This growth factor also augments the mRNA transcription levels and number of LH receptors [18,19].

The biological action of IGF-I is mediated by a transmembranal type I receptor observed in several species of mammals [23–26]. IGF-I null mutation experiments have established the crucial role of this gene for Leydig cell function and reproduction in the mouse; testes were reduced in size and they possessed less and smaller number of Leydig cells. Spermatogenesis was sustained at 18% and plasma testosterone (T) levels were lower than those observed in wild mice [12]. In spite of the information available, the role of IGF-I during embryonic gonadal development in

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the mouse is poorly understood. The present study examines IGF-I mRNA and protein expression in the testis on days E10–E17. We also demonstrate the role of IGF-I as an intratesticular promoter of the key steroidogenic enzymes 17α-hydroxylase and 17β-hydroxysteroid dehydrogenase.

2. Materials and methods

2.1. Animals

CD1 adult mice, grown at the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, were maintained on a 12/12 h light–dark cycle with access to food and water ad libitum. Pregnant mice were obtained by caging four females with one male overnight, and the morning the vaginal plug was found was counted as day E0. Pregnant mice were killed by cervical dislocation on days E10–E17, embryos were collected by hysterectomy and the gonads were removed, separated from the mesonephros and processed for the different studies.

2.2. Embryonic sex identification

Chromosomal sex of embryos on days E10 and E11 was identified by PCR analysis of the Sry gene present only in the Y chromosome [27], using somatic tissue DNA. Mouse embryos were staged according to limb development [28] as follows: day E10 (stages 1 and 2), and day E11 (stages 3 and 4). On days E12–E17, gonadal sex was established by the presence of highly vascularized testis cords, whereas their absence was related to ovarian development [29].

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR) experiments

Total RNA from pairs of embryonic testes was purified by the guanidinium thiocyanate–phenol–chloroform method [30]. Isolated RNA was resuspended in 5 μl of DEPC-treated water, denatured at 90 °C for 5 min, and reverse transcribed in a total volume of 20 μl, containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1 mM of each deoxynucleotide triphosphate, 1 unit of RNase inhibitor, 2.5 μM of random hexamers and 2.5 units of murine leukemia virus reverse transcriptase. The reaction mixture was incubated at 25 °C for 10 min and subsequently at 42 °C for 1 h. The enzyme was then inactivated at 99 °C for 5 min. In order to analyze IGF-I gene expression, we designed specific primers of 25 bp of length for RT-PCR experiments in a 5′ → 3′ (sense and antisense) order as follows: constitutive gene hypoxanthine phosphoribosyl transferase (Hprt)—CCTGCTGGATTACATTAAAGCACTG and GTCAAGGGCATATCCAACAACAAAC [31]; 5′ → 3′ IGF-I—ACCAGAGACCCTTTGCGGGGCT and AAGTGTACTTCCTTCTGAGTCT [32] sense and antisense, respectively. Oligonucleotides were synthesized by Life Technologies (Gaithersburg, MD). PCR was performed with a Perkin-Elmer Cetus Thermocycler (model 9600) in 100 μl total volume including 20 μl of cDNA, 40 pmol of each primer, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3) and 2.5 units of AmpliTaq DNA polymerase (Applied Biosystems, Branchburg, NY). Negative controls were performed without reverse transcriptase. Thirty-five basic cycles were performed as follows: denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s and DNA synthesis at 72 °C for 30 s. In the first cycle, denaturation was continued for 2 min and in the last cycle, extension was performed for 10 min. Subsequently, 10 μl of PCR amplification product of each reaction mixture were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining [33]. A low molecular DNA mass ladder was used as standard marker (Life Technologies Gaithersburg, MD). The expected fragment sizes were 396 and 352 bp for Hprt and IGF-I, respectively. Each experiment was performed three to five times for each stage studied. The RT-PCR products of IGF-I and Hprt gene expression were analyzed by densitometry using the AMBIS (Radio Analytic Image System Inc., San Diego, CA). Results are expressed as arbitrary units of the ratio between the two mRNA levels. IGF-I RT-PCR amplified product identity was established by restriction enzyme analysis with *Bsm*I (Life Technologies, Gaithersburg, MD). IGF-I was also hybridized with the IGF-I (pmigf 1–2) probe.

2.4. Western blot studies

Testes were homogenized in 250 mM Tris–HCl (pH 6.8) with a protease inhibitor cocktail (Roche Molecular Biochemicals, UK) and centrifuged at 1500 rpm for 5 min. The supernatant was recovered and protein concentration was determined according to techniques described elsewhere. Samples of 10 μg were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [34] and electrophoretically transferred to a PVDF membrane in 250 mM Tris–HCl (pH 8.3), 192 mM glycine and 20% methanol [35]. Unspecific signals were blocked with 3% bovine serum albumin overnight at 4 °C. The membrane was incubated with anti-somatomedin-C/IGF-I rabbit anti-serum NIH-UB-4 (1:3000) for 18 h at 4 °C (a kind gift of Drs. L. Underwood and J.J. van Wyck, obtained through The National Hormone and Pituitary Program of the NIH, Bethesda, MD) and rinsed with 10 mM Tris–HCl (pH 7.4), 0.9% NaCl and 0.1% Tween 20, and a secondary antibody–goat anti-rabbit IgG horseradish peroxidase conjugate (1:1000; Vector Laboratories, USA) were added for 18 h at 4 °C followed by color development with 4-chloro-1-naphtol and hydrogen peroxide. [36].

2.5. Endocrine activity of the isolated Leydig cell

Immunocytochemistry experiments from our laboratory suggest that whole embryonic testicular cells produce IGF-I

(unpublished data). In order to exclude paracrine effects of the IGF-I produced by other testicular cell types, we evaluated the IGF-I effect on isolated Leydig cells. Ten pairs of gonads from days E11 to E13 and eight pairs of gonads from days E14 to E17 were dissected and Leydig cells were separated according to the technique by Habert and Brignaschi [37] with slight modifications [40] and then cultured for 1 day in MEM and 0.4% fetal calf serum (1:1) to allow cells to attach. On the following days (D1–D5), cells were cultured in serum-free medium as described before [38], in the presence or absence of 50 or 100 ng of IGF-I (BACHEM Bioscience Inc., Torrance, CA) and 100 nM cAMP. Subsequently, 17α-hydroxyprogesterone activity was assayed by radioimmunoassay. Each experiment was performed five times.

2.6. Secretory activity of whole male gonads

Two pairs of testes were dissected from days E10 to E17, cut in half with a fine needle and incubated at 37 °C for 48 h in 5% CO_2 in a rotating tube containing 1000 μl of MEM supplemented as described before [38] in the presence or absence of 50 or 100 ng of IGF-I. Progesterone was added to the culture medium at a final concentration of 1 μg/ml. T production was assayed with T and 17α-progesterone kit (Diagnostic Systems Laboratory Inc., USA). The sensitivity limit of the assays was 0.1 ng/ml. We assumed that secreted 17α-hydroxyprogesterone and T in the culture medium represent steroidogenic activity of the whole testis. To assay explant viability after culture, 3β-hydroxysteroid dehydrogenase/Δ^4–Δ^5 isomerase activity was analyzed histochemically using dehydroepiandrosterone as substrate [38].

2.7. Statistical analysis

All values are means (± S.E.M.). The significance of differences between mean values of control group and IGF-I experimental groups were analyzed by Dunnet's test [39] and as well as paired Student's *t*-test ($P < 0.0001$). Variance analysis was applied with a block design and the post-hoc Tukey's test when several groups were compared. All experimental data were analyzed with SAS 6.02 program (SAS Institute Inc., Cary, NC 711, USA).

3. Results

3.1. IGF-I is present in the presumptive testis and undifferentiated male gonad

IGF-I gene expression was first observed in the presumptive testes in the urogenital ridge on day E10. On day E11, a still undifferentiated stage, IGF-I transcription levels were similar to those detected on day E10 (Fig. 1A). IGF-I transcription levels markedly diminished on previous stages

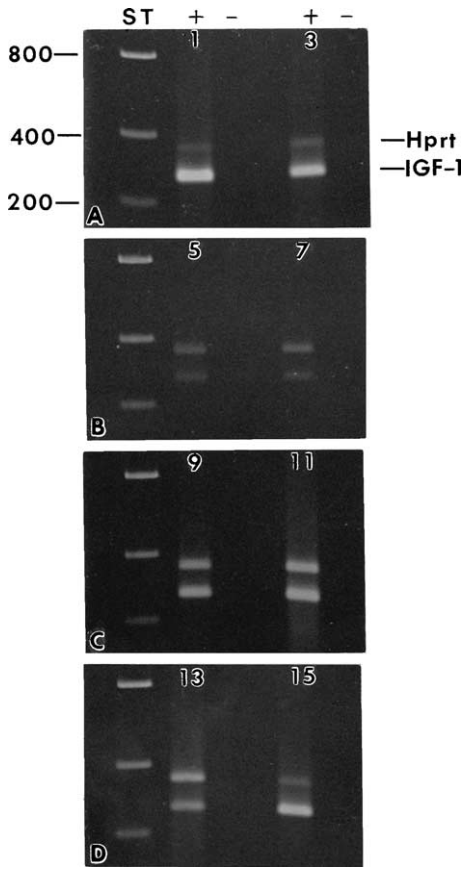

Fig. 1. Ontogenetic expression of IGF-I gene in the testis: (A) expression levels of the IGF-I gene on day E10 (urogenital ridge stage, lane 1) and on day E11 (undifferentiated gonad stage, lane 3); (B) transcription levels of the IGF-I mRNA from days E12 to E13 (testicular differentiation stage, lanes 5 and 7); (C) on days E14 and E15 (early fetal period testis, lanes 9 and 11); (D) on days E16 and E17 (late fetal period testis, lanes 13 and 15). RT-PCR experiments were performed in the presence (+) and absence (−) of reverse transcriptase. ST: molecular weight standard. Expected fragment sizes for Hprt and IGF-I amplified genes are 396 and 352 bp, respectively.

compared to days E12 and E13 when sexual differentiation takes place (Fig. 1B). On day E14, the IGF-I gene displayed higher transcription levels than those present from days E10 to E13. The highest IGF-I mRNA expression was found on day E15. (Fig. 1C). Levels observed on day E16 were slightly lower to those detected on day E14. On day E17, IGF-I gene expression was similar to day E11 (Fig. 1C and D). Hprt gene expression displayed similar intensity from days E10 to E13 and its transcription level increased from days E14 to E16 (Fig. 1D). On day E17, Hprt mRNA expression diminished to the levels found on days E10 and E11 (Fig. 1D). Densitometric analysis of the IGF-I/Hprt mRNA gene ratio corroborated the observed differential expression (Fig. 2). IGF-I gene expression was also found in the isolated mesonephros throughout testicular development on days E10–E17 (data not shown).

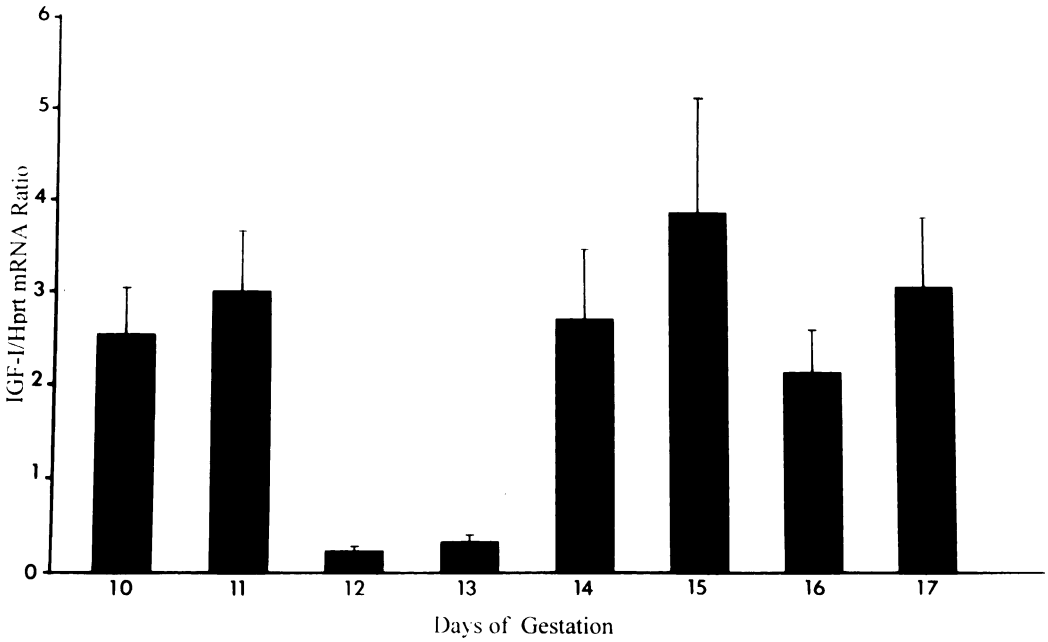

Fig. 2. Embryonic expression of the IGF-I gene in the testis. IGF-I/Hprt mRNA ratios are shown. Bars represent the mean ± S.D. of five separate experiments.

3.2. Several IGF-I species are detected within the embryonic male gonad

Western blot experiments demonstrated the presence of five proteins with molecular weights from 100 to 31 kDa in the embryonic testis during testicular development. A fine band of 31 kDa was present from days E12 to E13 and its expression increased on day E14. Hereafter, observed expression levels were similar in all stages examined (Fig. 3).

3.3. IGF-I enhances prenatal testosterone production

Experiments with 50 ng/ml of IGF-I showed no effect on T production in the testis and T levels were similar to the control group after 48 h of culture. On day E10, in the testes cultured with 100 ng of IGF-I, T levels were undetectable. The beginning of T output was on day E11, it increased three-fold thereafter with the highest levels on day E17 (Fig. 4). The T assay revealed 3.40% (5α-dihydrotestosterone), 2.00% (11-oxotestosterone) and 0.20% (5α-androstene-3α,17β-diol).

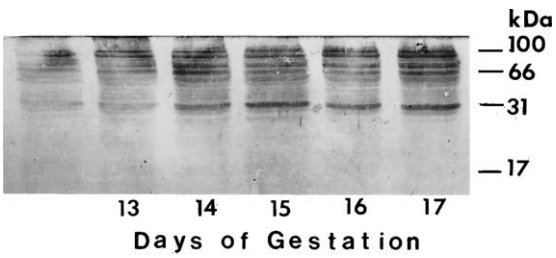

Fig. 3. Different bands of IGF-I protein are produced within the testis during testicular development.

3.4. IGF-I regulates 17α-hydroxylase expression in the testis

Experiments with 50 ng/ml of IGF-I had no effect on 17α-hydroxyprogesterone secretion in the testis. In testes cultured with 100 ng of IGF-I, 17α-hydroxyprogesterone was undetectable on day E10. In testes cultured with 100 ng of IGF-I, onset of 17α-hydroxyprogesterone production was

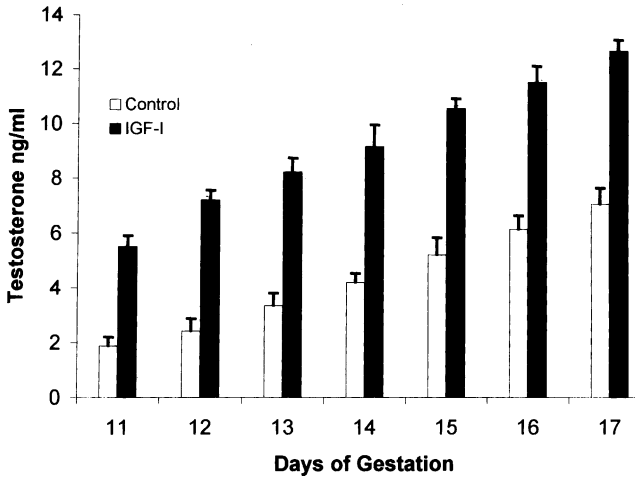

Fig. 4. IGF-I promotes testosterone secretion in the embryonic testis (radioimmunoassay experiments). Bars represent mean ± S.D. of five to six separate experiments ($P < 0.0001$).

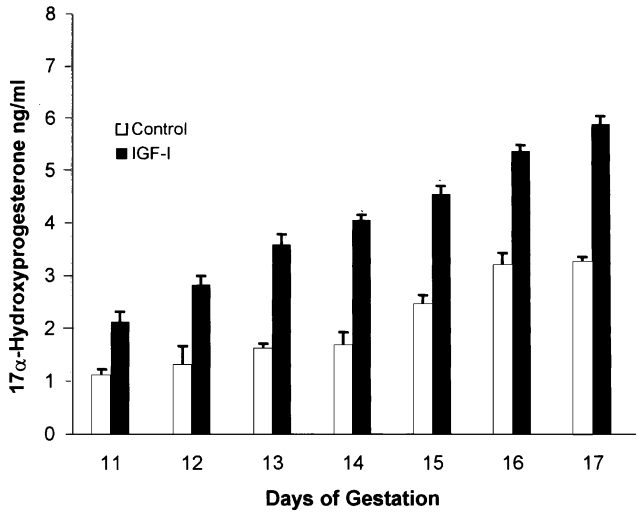

Fig. 5. Effect of the IGF-I on 17α-hydroxyprogesterone in the male gonad. Bars represent mean ± S.D. of five to six separate experiments ($P < 0.0001$).

on day E11 and its levels increased throughout the stages analyzed compared with the control group (Fig. 5).

3.5. Synergistic effect of IGF-I and cAMP on 17α-hydroxylase regulation

Leydig cells cultured in the presence of 100 nM of $(Bu)_2$cAMP plus IGF-I increased 17α-hydroxyprogesterone secretion three-fold compared with IGF-I and $(Bu)_2$cAMP group alone (Fig. 6). Observed secretion values of this steroid rose according to days of culture (data not shown).

3.6. Statistical significance

Statistical analysis revealed significant differences in 17α-hydroxyprogesterone (Fig. 3) and testosterone (Fig. 4) production in all studied stages ($t = 9.23$ and 24.68, respectively; $P < 0.0001$). Multiple comparison Tukey's test showed significant differences, $P < 0.05$ in all experimental groups (Fig. 6).

4. Discussion

The present investigation demonstrates IGF-I mRNA and protein expression as well as the role of IGF-I as a modulator of the key steroidogenic enzymes P450 c17 and 17β-hydroxysteroid dehydrogenase during testicular development. Onset of IGF-I gene expression in male gonads was on day E10 when the genital ridge initiates morphogenesis [29]. Its presence at this early stage in the mouse appears to be involved in the proliferation and differentiation of Sertoli and Leydig cell precursors in the gonad during development of the embryonic testes. Preliminary results obtained by our group support this observation (Villalpando et al., unpublished data).

Conversely, IGF-I knockout mice primarily exhibit defects in postnatal functioning and population of Leydig cells, which is thought to be different from the population involved in sexual differentiation in utero [40]. Virilization of Wolffian duct derivates appears to be normal in IGF-I knockout mice, although the structures fail to undergo the secondary growth and differentiation that normally occur posnatally [12]. Apparent normal development of fetal Leydig cells in knockout mice in utero suggest that endocrine activity of

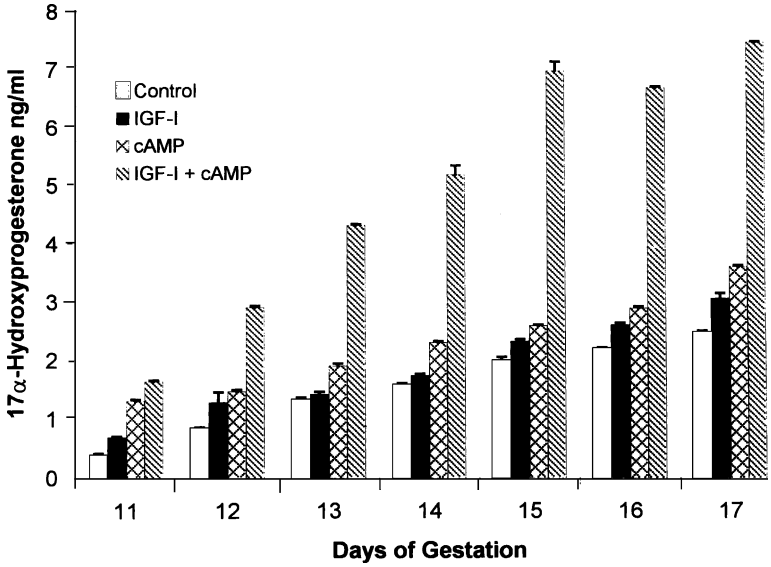

Fig. 6. Synergistic effect of the IGF-I plus $(Bu)_2$cAMP on 17α-hydroxyprogesterone secretion in Leydig cells. Bars represent mean ± S.D. of five to six independent experiments ($P < 0.05$).

testes is controlled by other factors besides IGF-I. In these mice, failure of postnatal Leydig cells to produce normal levels of testosterone could be due to mechanisms involved in postnatal control of steroidogenesis at this stage that are different from those in the prenatal period.

It was found that IGF-I gene expression diminishes on days E12 and E13, when testis sex differentiation occurs. Lesser expression of IGF-I transcripts found at this stage may be because IGF-I being translated to protein induces morphological and physiological differentiation of Leydig cell precursors. Previous experiments performed in the rat during the fetal period sustain this finding [8].

We observed the presence of different IGF-I bands demonstrating that several proteins are produced within the testis during early development. Northern blot experiments reported that several IGF-I transcript species are present in the rat testis during the fetal period [8]. Interesting investigations about gene expression of key steroidogenic enzymes show that protein expression is low compared with mRNA expression [40]. In the present study, discrepancies observed between lower IGF-I mRNA levels and comparable IGF-I protein expression found throughout testicular development could be explained by either postransductional regulation or stabilization of the IGF-I protein.

In vitro studies have demonstrated that the IGF-I gene postnatally regulates the rate of expression of genes encoding key steroidogenic enzymes involved in the biotransformation of steroid hormones in the ovary and testis of various mammalian species [15–22]. Endocrine activity in the male mouse gonad can be detected by the presence of transcripts for P450 c17 beginning on day E12 [41], and by 17β-hydroxysteroid dehydrogenase activity present at even earlier developmental stages [42] in the mouse. We observed that IGF-I regulates enzyme activity of P450 c17 and 17β-hydroxysteroid dehydrogenase prenatally in vitro demonstrating the role of this growth factor in the regulation of endocrine activity in the embryonic testis. Male gonads cultured in the presence of IGF-I increased T production during testicular development and the highest levels were detected on day E17. This observation can be explained by either a larger number of Leydig cell precursors, as previously described in the rat [43], or higher efficiency of key steroidogenic enzymatic machinery present at this developmental stage in the testis.

In the current study, we did not find an effect of IGF-I on testosterone and 17α-hydroxyprogesterone production of cultured mice testis with 50 ng of IGF-I at any analyzed stage. However, several studies have shown that IGF-I binding proteins (IGF-I BPs) inhibit the positive effects of the IGF-I on Leydig cell function [44]. Thus, it is likely that embryonic testicular cells contain higher endogenous IGF-I BPs that inhibit the IGF-I effect. The finding that only 100 ng of IGF-I affect sexual steroid secretion could be explained by higher IGF-I concentration that overcomes the effect of the IGF-I BPs leading to activation of the steroidogenic enzymes and allowing IGF-I to activate other genes leading to higher steroid hormone secretion. Alternatively, higher levels of TGF-β factor produced by embryonic testis could inhibit enzyme activity of fetal Leydig cells in the mouse, since it has been proposed that TGF-β inhibits testosterone production in testicular dispersed cells [45].

In this study, we must also take into account that 100 ng of IGF-I is a very high dose and even though we did not measure IGF-I in mouse testis it has been reported that fetal rat testis produce 18.7 ± 2.5 pg per testis. [8]. Thus, it is likely that a higher dose of IGF-I could induce steroidogenic enzymes to metabolize cholesterol and secrete other metabolites which could in turn be used as substrate to produce other testicular hormones.

In earlier studies on the regulation of P450 17α-hydroxyprogesterone, it was found that cAMP is necessary for the de novo synthesis of this enzyme in normal mouse cells [40]. We found that fetal testicular cells cultured in the presence of $(Bu)_2$cAMP plus IGF-I showed increased levels of progesterone production. This observation suggests that IGF-I promotes a signaling mechanism in the embryonic secretory cells involving the protein kinase pathway.

Finally, it is hypothesized that during testicular development, IGF-I acts as an autocrine factor inducing proliferation of somatic cells [8] in the presumptive male gonad at early developmental stages, and promoting physiological differentiation of mesenchymal cells into Leydig cells. Further experiments are required to gain insight into this important process for reproduction and male fertility.

In conclusion, we demonstrate for the first time that the mouse fetal testis produces IGF-I factor in various critical stages of gonadal development. In addition, we show that IGF-I promotes activity of key steroidogenic enzymes of the fetal testis in utero. We also demonstrate that IGF-I enhances expression of 17α-hydroxylase enzyme in the presence of $(Bu)_2$cAMP, suggesting that the IGF-I transduction signal in the testis involves the protein kinase pathway.

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