Insulin-Like Growth Factor 1 Is Expressed in Mouse Developing Testis and Regulates Somatic Cell Proliferation

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Testicular development occurs prenatally in mammals. The developmental underlying mechanism is only partially understood. The aim of the present investigation was to study the expression of the gene coding for *insulin-like growth factor 1* (*Igf-1*) and *Igf-1* type 1 receptor (*Igf-1r*) and their respective proteins in mouse Sertoli and Leydig cells at gestation day 12 (E12)–E18. Moreover, we sought to determine the effect of IGF-1 on the proliferation of both cell types and to establish the signal transduction mechanism involved in the IGF-1 pathway. Transcripts for the *Igf-1* and *Igf-1r* genes were found in Sertoli and Leydig cells from E12–E18. Highest IGF-1 and IGF-Ir protein expression levels were found in both cell types at E18. Exogenous IGF-1 administration increased Sertoli and Leydig cell proliferation at E14–E18 *in vitro*. Inhibition of the pathway mitogen-activated extracellular signal-regulated protein kinase (MEK) 1/2 with UO126 diminished the proliferation of the Sertoli and Leydig cells *in vitro*. We propose that IGF-1 and IGF-1r regulate Sertoli and Leydig cell proliferation through the MEK/extracellular-signal-regulated kinase (ERK) 1/2 signal transduction pathway, leading to development and growth of the mouse embryonic testis. Exp Biol Med 233:419–426, 2008

Key words: IGF-1; testis; proliferation; Leydig cell; Sertoli cell

Introduction

During mouse gonadal development, the primary testis-determining *Sry* gene is necessary to induce down-regulation of other important genes leading to the male differentiation pathway (1). The crucial role for the insulin and insulin-related proteins, such as IGF-1, was established for mouse testicular differentiation (2, 3). Targeting experiments demonstrated the importance of IGF-1 for the development and fate of the mouse male gonad (4). It has been proposed that locally produced IGF-1 plays both an autocrine and paracrine function in the adult testis (5–7); however, the precise role of IGF-1 in the context of the developing testis remains to be clarified. One of the most studied aspects of IGF-1 is its function as a regulator of the expression of key steroidogenic enzymes in the embryonic and postnatal testes of mammals (8–12). IGF-1 is expressed in the testis of various species in the adult Leydig and Sertoli cells (5–7). The biological action of IGF-1 is elicited through a *trans*-membrane receptor, which is a tetrameric complex composed of two α and two β subunits (13). The mRNA for IGF-1r is found in the rat spermatocytes and spermatids of the adult testis (14–16). IGF-1r expression has also been described in rat testis (17). On the other hand, it is known that proliferation is a process finely regulated by oncogenes, proteins, cyclin-dependent kinase complexes, and growth factors such as IGF-1 (18–20). Previous investigations demonstrated that IGF-1 activates transition stages of various cellular types in adult tissues (12, 21). However, the role of IGF-1 as regulator of Leydig and Sertoli cell proliferation in the developing testis remains controversial at the present time. To gain insight into the role of IGF-1 in the mouse prenatal testis, we studied *Igf-1* and *Igf-1r* gene and protein expression, as well as the IGF-1 effect on Sertoli and Leydig cell proliferation, in mice developing testis.

Material and Methods

Animal Breeding and Generation of Embryos.

CD1 mice were maintained and handled according to the National Institutes of Health Guide for the Care and Use of

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Laboratory Animals (NIH 85–23, revised 1985). All methods used in this investigation were approved by the Internal Council and the Animal Care Committee of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. Four adult, eight-week-old female breeders were caged with a male of the same age. Females with a vaginal plug on the day subsequent to mating were considered to be at day 0 of pregnancy. Pregnant females at 12, 14, 16, and 18 days of pregnancy were sacrificed by cervical dislocation. The embryos were dissected out from the uterus and immediately sacrificed. The testes were isolated and collected in Dulbecco's modified Eagle medium (D-MEM).

Identification of Testis. Mouse embryos of the above-mentioned ages were staged according to limb development as previously described (22) and are referred to hereafter as E12 (onset of testicular differentiation), E14 (beginning of growth), E16 (fetal stage), and E18 (perinatal period). Male gonads were identified by the presence of the highly vascularized testis as shown under a stereo microscope (23).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. To determine *Igf-1* and *Igf-1r* expression, testes at stages E12–E18 were separated under a stereo microscope and frozen at –20°C. Total RNA from pooled testes was purified using Trizol-guanidinium-thiocyanate (Life Technologies, Gaithersburg, MD). One microgram of total RNA was reverse transcribed with 200 units of M-MLV reverse transcriptase (Applied Biosystems, Branchburg, NJ) in a total volume of 50 µl. *Igf-1* and *Igf-1r* receptor genes were amplified by RT-PCR according to the protocol provided by the manufacturer. Oligonucleotide sequence primers were synthesized by Life Technologies and used at 100 pg. The oligonucleotide sequences for *Igf-1* and the β-*actin* oligonucleotide were those previously described (8) for *Igf-Ir* as follows: sense 5′CACTCTGT-ACCGCATCGATATC3′, antisense 5′GGCAACCGGCA-GAGCAATGATC3′. A total of 40 cycles were used to amplify the *Igf-Ir:* 94°C 60-sec denaturation; 58°C 90-sec annealing, and elongation at 72°C for 2 mins or 20 mins in the last cycle. RT-PCR products were separated by electrophoresis and visualized by ethidium bromide staining. A low DNA Mass Ladder was used as a standard marker (Life Technologies). In each experiment, negative control reactions were performed under the same experimental conditions in the absence of reverse transcriptase. At least three independent experiments using four male gonads at E12–E18 were performed.

Determination of Endogenous IGF-1 in the Testis. The amount of endogenous IGF-1 produced by testicular cells was analyzed by radioimmunoassay. Male gonads from E12–E18 were isolated, and 40 testes were pooled for each developmental stage analyzed. Briefly, samples were homogenized, IGF-1 was separated from its binding proteins by acid ethanol extraction as described before (24), and samples were processed for radioimmunoassay according the manufacturer's protocol using an IGF-1 DSL, 5600 extraction kit (Diagnostic Systems Laboratories, Webster, TX). The sensitivity limit of the assay was 1 pg, and the interassay coefficient was 8.7 for a sample containing 1 pg/ml. The IGF-1 production was measured in developing testis in three independent experiments, with freshly obtained testes isolated for each developmental stage.

Isolation of Leydig and Sertoli Cells. The embryonic testes at E16–E18 were isolated and placed in D-MEM and processed independently according to the technique described before (25), with slight modifications. Briefly, testes were rinsed twice with Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS) and incubated with 0.2 mg/ml collagenase-dispase and 0.1 mg/ml DNase I (Roche, Indianapolis, IN) for 10–15 mins at 37(C according to their developmental stage. Gonads were then mechanically disaggregated with a sterile, siliconized Pasteur pipette with Sigmacote (Sigma, St. Louis, MO). The enzyme was inhibited with 0.2% trypsin inhibitor (GIBCO, Carlsbad, CA), and cells were allowed to sediment at room temperature for 15 mins. The supernatant containing Leydig cells was separated and centrifuged at 1894 *g* for 5 mins. The sedimented fraction containing Sertoli cells was treated with 0.1% trypsin for 1–3 mins, and enzyme activity was inhibited. The pellet was resuspended in D-MEM cultured medium supplemented as described before (8). To synchronize cells 1×10^6, Leydig and Sertoli cells were incubated with 10% of bovine fetal serum. Sertoli cells were seeded in F12:D–MEM 1:1. After 24 hrs of culture, the medium was removed and the cells were cultured with fresh medium, without bovine fetal serum, to eliminate germ cells (26). The following day, Sertoli and Leydig cells were cultured in the presence of 10 µm of bromodeoxyuridine (BrdU) and 100 ng of recombinant IGF-1 for 24 hrs. Cells were normalized, taking into consideration BrdU-unlabeled and -labeled cells, and results were expressed as percentage of labeled Sertoli and Leydig cells. Each experiment was performed at least five times in each stage described.

Identification of Sertoli and Leydig Cells. The purity of Sertoli and Leydig cells fraction was established by the histochemical detection of 3β-hydroxysteroid dehydrogenase activity (3β-HSD) according the technique described previously (27). Positive cells to 3β-HSD revealed that 96% of the cells in the preparations were Leydig cells. Myoid cell percentage was established by immunocytochemistry with a muscle alpha actin antibody (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom). Immunostaining revealed the presence of $<1\%$ of myoid cells. Sertoli cells were identified by Müllerian inhibiting substance expression (R & D System, Minneapolis, MN), and the presence of reproductive cells was excluded by phosphatase alkaline staining (28). No reproductive cells were observed in Sertoli cells cultured in the absence of bovine fetal serum.

Immunocytochemistry. Immunodetection of IGF-1 and the α and β subunits of IGF-1r was performed in

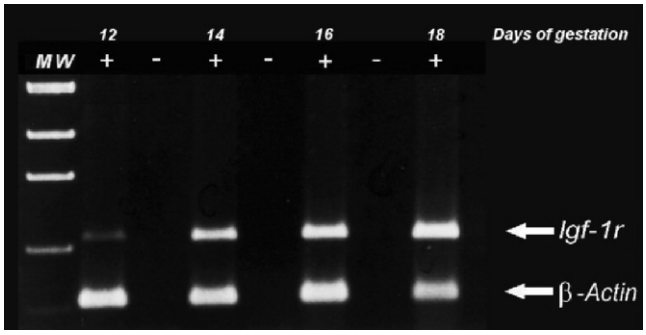

Figure 1. *Igf-Ir* expression analyzed by RT-PCR in the mouse testis, in the presence (+) and absence (–) of reverse transcriptase. MW, molecular weight standard. Expected fragment sizes for *Igf-1r* and β-*actin* amplified genes were 442 and 352 bp, respectively.

cultured Sertoli and Leydig cells grown on round coverslips coated with 0.2% collagen by indirect immunoperoxidase activity. Endogenous peroxidase was blocked with 0.3% H_2O_2, and unspecific staining was blocked with 5% bovine serum albumin. Samples were rinsed and incubated overnight at room temperature with an anti-rabbit IGF-1 antibody at a 1:20 dilution or an anti-goat IGF-1r antibody (R & D System) at a 1:10 dilution. Immunostaining was performed following the manufacturer's instructions from ABC Kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was revealed with 0.3% H_2O_2 using 3,3′ diaminobenzidine tetrachloride, and the reaction was stopped with distilled water. Primary antibody for IGF-1 and IGF-Ir was omitted as a negative control.

Flow Cytometry Cell Cycle of Somatic Testicular Cells. Flow cytometry cell cycle analysis in Leydig and Sertoli cells at 18 days of gestation was performed essentially as previously described (29), with minor modifications. Briefly, Sertoli and Leydig cells (1×10^6) in 300 µl of PBS were permeabilized by adding 0.75 µl of 10% Triton X-100 in PBS. Then, 30 µl of 10 mg/ml RNAse solution was added simultaneously to the cell suspension in order to eliminate propidium iodide staining of double-stranded RNA. After 30 mins of incubation at 37°C, 5 µl of a 100-µg/ml solution of propidium iodide was added to the cell suspensions. Cells were incubated at 37°C for 30 mins and were finally fixed by adding 100 µl of 10% paraformaldehyde in PBS. Flow cytometric analysis was performed using a FACScan apparatus (Becton Dickinson, Franklin Lakes, NJ) with logarithmic amplification of the side scatter and forward scatter parameters. Propidium iodide fluorescence was detected using the FL-2 channel set at 564 with linear amplification. A standard of DNA content was obtained by serum-starving Leydig cells for 4 days. To evaluate the effect of IGF-1 on the cell cycle, Leydig or Sertoli cells were treated with and without 100 ng/ml of recombinant IGF-1 for 24 hrs, in the presence or absence of 30 µ*M* of the MEK 1/2 UO126 inhibitor.

Statistical Analysis. The effect of IGF-1 on Sertoli and Leydig cell proliferation was analyzed with variance analysis followed by Student's *t* test. The IGF-1 produced in the male gonad was analyzed with one-way ANOVA followed by the Tukey test with the program SAS 6.02 (SAS Institute Inc., Cary, NC), with significance set at $P < 0.05$.

Results

Transcripts for *Igf-1* and *Igf-1r* Genes are Expressed in Somatic Cells of the Mouse Embryonic Testis. The onset of *Igf-1r* mRNA expression was at E12; at this stage, we detected a faint expression in the entire mouse testis. At E14, transcripts for the *Igf-1r* gene were moderately higher than the expression levels found in the previous stage. *Igf-1r* gene expression increased according to developmental stages in the testis at E16–E18 (Fig. 1). Lengths of the amplified genes were 442 and 352 bp for the *Igf-1r* and β-*actin* genes, respectively.

Testicular Cells Expressed IGF-1 and IGF-1r. Faint staining for the IGF-1 protein was observed in the cytoplasm of Leydig and Sertoli cells at E12 and E14. Expression of IGF-1 increased moderately at E16, and

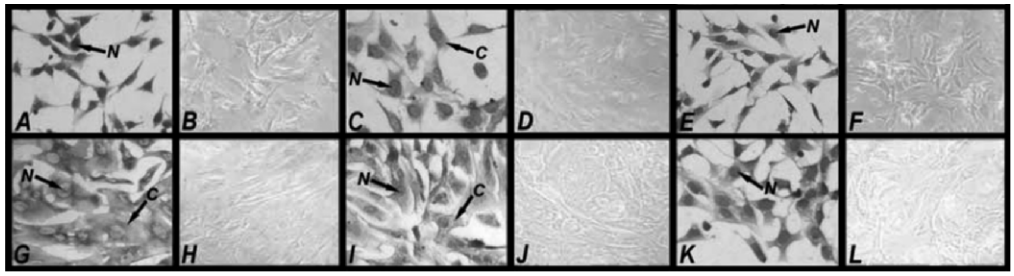

Figure 2. *Igf-I* and *Igf-1r* gene and protein expression in mouse testicular cells. A. IGF-1 moderate staining was located in the cytoplasm of Leydig cells, whereas higher immunostaining was observed in the nucleus (N). ×20. B. Control of IGF-I staining. Note the absence of staining in the cultured Leydig cells. ×20. C. Shows staining for β subunit of IGF-1r . The β subunit was also found in the nucleus (N), while fainter staining was seen in the cytoplasm (C) of Leydig cells. ×40. D. No immunostaining was found in the absence of the first antibody. ×20. E. The α subunit of the type 1 receptor was also seen in the nucleus (N) of Leydig cells. ×20. F. In the control group, an immunostaining pattern was absent in the cells. ×20. G: IGF-1β receptor expression in the cytoplasm of Sertoli cells can be observed. ×40. H. No immunoreaction was detected in the cytoplasm of Sertoli cells. ×20. I. Sertoli cells moderately expressed the β subunit receptor in the nucleus (N). ×40. J No staining was seen in the cytoplasm or nucleus of this cell type. ×40. K. Expression of IGF-I α subunit receptor in the nucleus (N) of Sertoli cells. ×40. L. Note the absence of immunostaining in cultured Sertoli cells of the mouse testis. ×40.

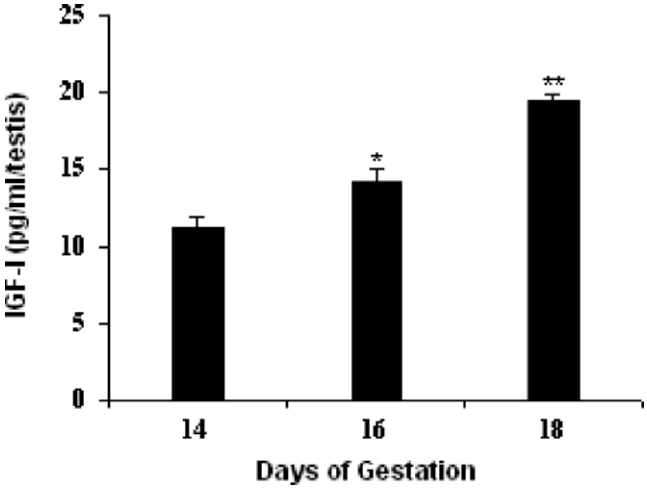

Figure 3. Endogenous IGF-1 production in the mouse testis analyzed by radioimmunoassay. The bars indicate the mean ± SE from 3 experiments. $^{*}P < 0.05$ by the Tukey test.

immunostaining at this stage was detected in the nucleus of Leydig cells (data not shown). At E18, the highest IGF-1 protein expression in this cell type was observed in the nucleus, while fainter staining was found in the cytoplasm (Fig. 2A; ×20). No immunostaining was found in the control cells (Fig. 2B; ×20). The α and β subunits of the type 1 receptor also exhibited high staining in the nucleus of Leydig cells (Figs. 2C–E, ×40 and ×20, respectively). Note the absence of expression of α and β subunit receptors of the type 1 receptor in the cells of the control group (Fig. 2D–F; ×20). IGF-1 expression in Sertoli cells was observed mainly in the cytoplasm (Fig. 2G; ×40). No staining was seen in the cytoplasm of these cells (Fig. 2H; ×20). The IGF-I β receptor subunit showed moderate staining in the nucleus of Sertoli cells, whereas higher immunostaining for the α subunit was detected in the nuclear compartment of these cell types (Fig. 2I–K; ×40). No immunostaining was seen in Sertoli cells in the absence of the α and β subunit antibodies (Fig. 2J–L; ×40).

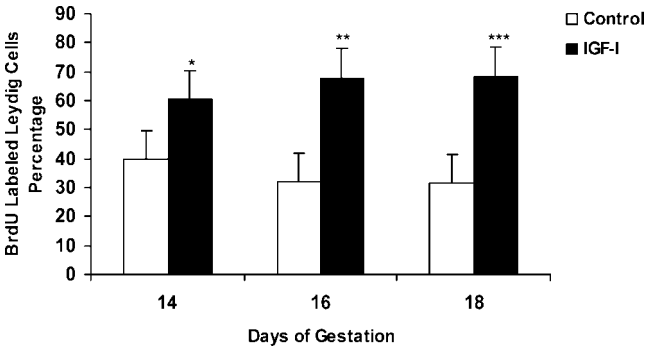

Figure 4. Proliferation of mouse Leydig cells analyzed by BrdU incorporation. Data represent the mean ± SE from five independent experiments. $^{*}P < 0.05$ IGF-1, compared with control group at 14 days of gestation. $^{**}P < 0.05$ compared with control group at 16 days of gestation. $^{***}P < 0.05$ compared with control group at 18 days of gestation by Student's *t* test.

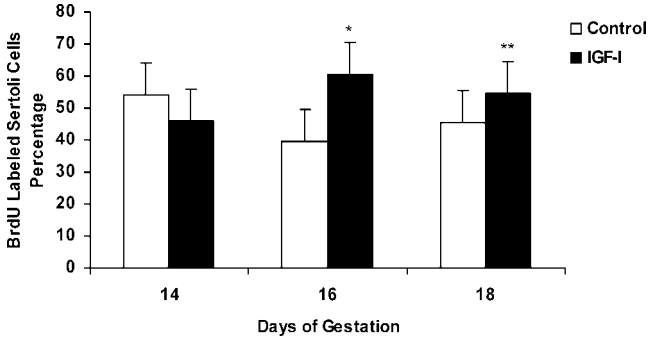

Figure 5. Proliferation of Sertoli cells analyzed by BrdU incorporation. The bars represent the mean ± SE from five independent experiments. $P < 0.05$ IGF-1 compared with the control group at 14 days of gestation. $^{*}P < 0.05$, IGF-1 compared with the control group at 16 days of gestation. $^{**}P < 0.05$, IGF-1 compared with the control group at 18 days of gestation, Student's *t* test.

Embryonic Mouse Testis Produced IGF-1. At E12, IGF-1 was undetectable in the testis by radioimmunoassay. In contrast, the amount of endogenous IGF-1 detected at E14 was 11 pg/ml, and its production increased to 14 pg/ml at E16. The highest value observed was 19 pg/ml at E18. The observed differences were statistically significant, with $^{*}P < 0.05$, with the Tukey test (Fig. 3).

IGF-1 Induced Leydig Cell Proliferation. At E12, we did not find any differences in proliferation between the control and IGF-1–treated Leydig cells by BrdU staining. At E14, 40% of the Leydig cells in the control group were BrdU-labeled, whereas in the IGF-1–treated cells, we observed 60% of BrdU incorporation. At E16, 32% of the Leydig cells in the control were BrdU-labeled, while 68% were BrdU labeled after IGF-1 treatment. At E18, the percentage of BrdU-positive cells in the control was 31%, while the percentage of labeled cells observed in the IGF-1–treated samples was 69% (Fig. 4). Statistical analysis revealed that differences found at E14 and E18 in the IGF-1–treated samples with respect to controls were statistically significant, *, **, $^{***}P < 0.05$, respectively, with Student's *t* test.

IGF-1 Promoted Sertoli Cell Proliferation. At E14, 54% of BrdU-positive Sertoli cells were found in the control group, whereas in the IGF-1–treated cells, we detected 46% of BrdU incorporation. At E16, 39% of Sertoli cells were BrdU-labeled in the control group, while 61% of the cells were BrdU-labeled in the group incubated with IGF-1. At E18, BrdU incorporation found in the control group was 46%, while the percentage of BrdU-positive cells observed in the IGF-1–treated samples was 54% (Fig. 5). Statistical analysis revealed that no statistically significant differences were observed at E14 in the control samples compared with IGF-1–treated cells. Differences found in the control group with respect to the IGF-1–treated group were statistically significant at E16–E18, *, $^{**}P < 0.05$, respectively, with Student's *t* test (Fig. 5).

Flow Cytometric Analysis of the Sertoli and Leydig Cell Cycle. In the absence of IGF-1, 64% of the

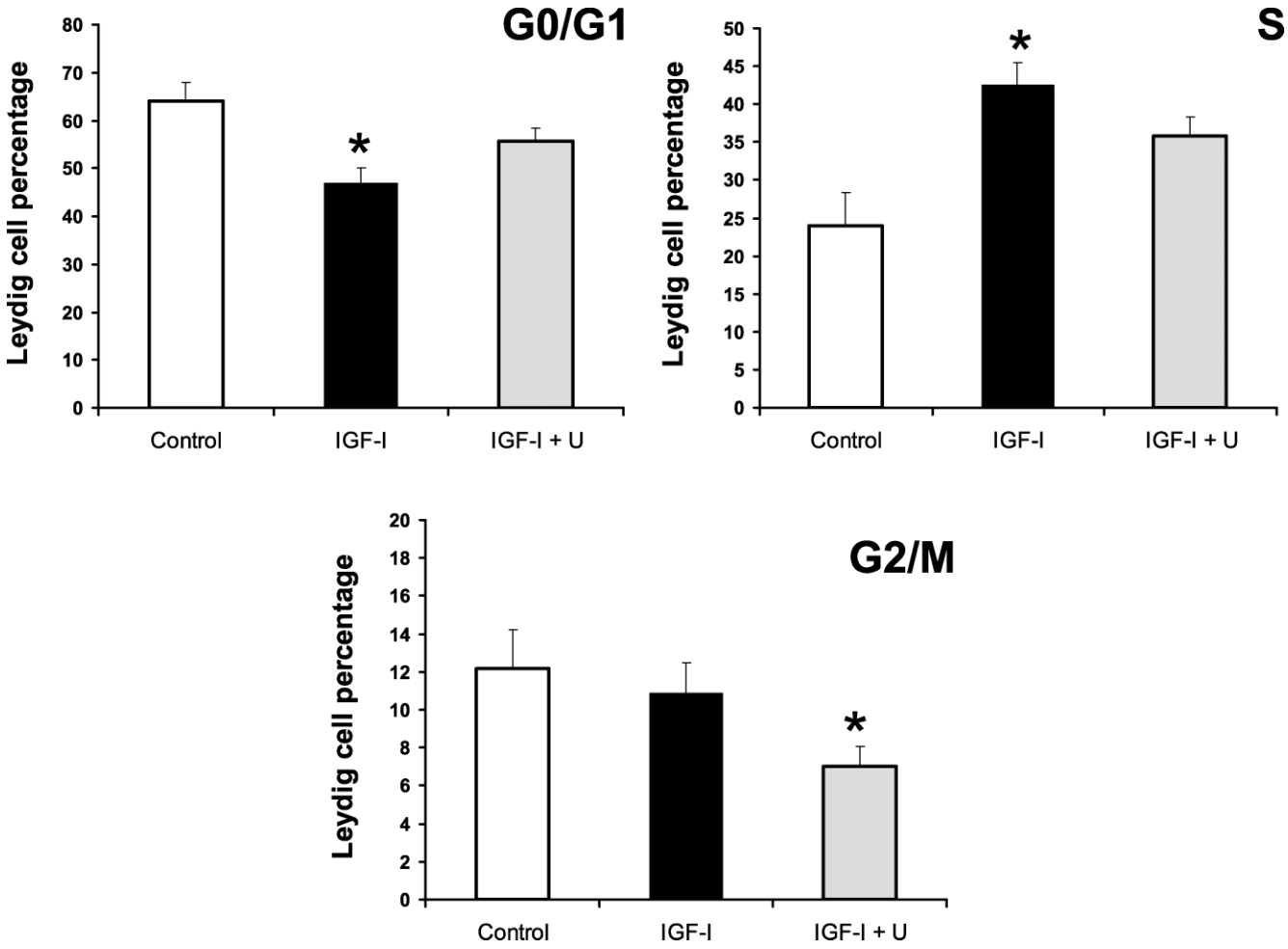

Figure 6. The effect of IGF-1 on the cell cycle of Leydig cells studied by flow cytometry. Treating cells with IGF-1 decreased the percentage of cells in the G0/G1 phase of the cell cycle, thus inducing a concomitant increase in the percentage of cells in either the S or G2/M phases. Inhibition of the MEK/ERK 1/2 pathway with the UO126 inhibitor reversed the IGF-1 effect. In the G0/G1 phase, $*P < 0.01$ for IGF-1 compared to control group. In the S phase, $*P < 0.007$ for IGF-1 compared to control group. In the G2/M phase, $*P < 0.02$, for IGF-1 compared to control group.

Leydig cells remained in the G0/G1 phase of the cell cycle; however, after IGF-1 treatment, this percentage was reduced to 47%. This difference from the control cells was statistically significant at $*P < 0.01$ (Fig. 6). The percentage of Leydig cells remaining in G0/G1 was 54% when cells were treated with the MEK/ERK inhibitor UO126 before IGF-1 stimulation. In the absence of IGF-1, only 24% of the Leydig cells were found in the S phase of the cell cycle. This percentage increased to 43% when Leydig cells were treated with IGF-1, and this difference was statistically significant at $*P < 0.007$. Leydig cells in the S phase were 35% when treated with UO126 before IGF-1 stimulation. No significant differences were found in the G2/M phase of the cell cycle between control and IGF-1–treated Leydig cells, 12% and 11%, respectively. However, the inhibition of MER/ERK 1/2 reduced the percentage of Leydig cells in G2/M to 7%, and this difference from the control cells was statistically significant at $*P < 0.02$ (Fig. 6).

The percentage of Sertoli cells in the G0/G1 phase of the cell cycle was 45% in the absence of IGF-1, and was reduced to 36% after IGF-1 stimulation. Treating Sertoli cells with UO126 before IGF-1 stimulation resulted in 51% of the cells remaining in the G0/G1 phase of the cell cycle (Fig. 7). In the absence of IGF-1, 33% of the Sertoli cells were in the S phase, and this percentage increased to 43% after IGF-1 stimulation. Treating Sertoli cells with UO126 before IGF-1 stimulation reduced the percentage of cells in the S phase to 32%. Differences from the control samples were statistically significant at $*P < 0.02$. In the absence of IGF-1, 13% of the Sertoli cells were in the G2/M phase of the cell cycle, and this percentage increased to 19% after IGF-1 stimulation. Treating Sertoli cells with UO126 before IGF-1 stimulation reduced the percentage of cells in the G2/M phase to 14%. Differences from control samples were statistically significant at $*P < 0.02$.

Administration of the UO126 in the absence of IGF-1 had no effect on the Sertoli and Leydig cell cycle (data not shown).

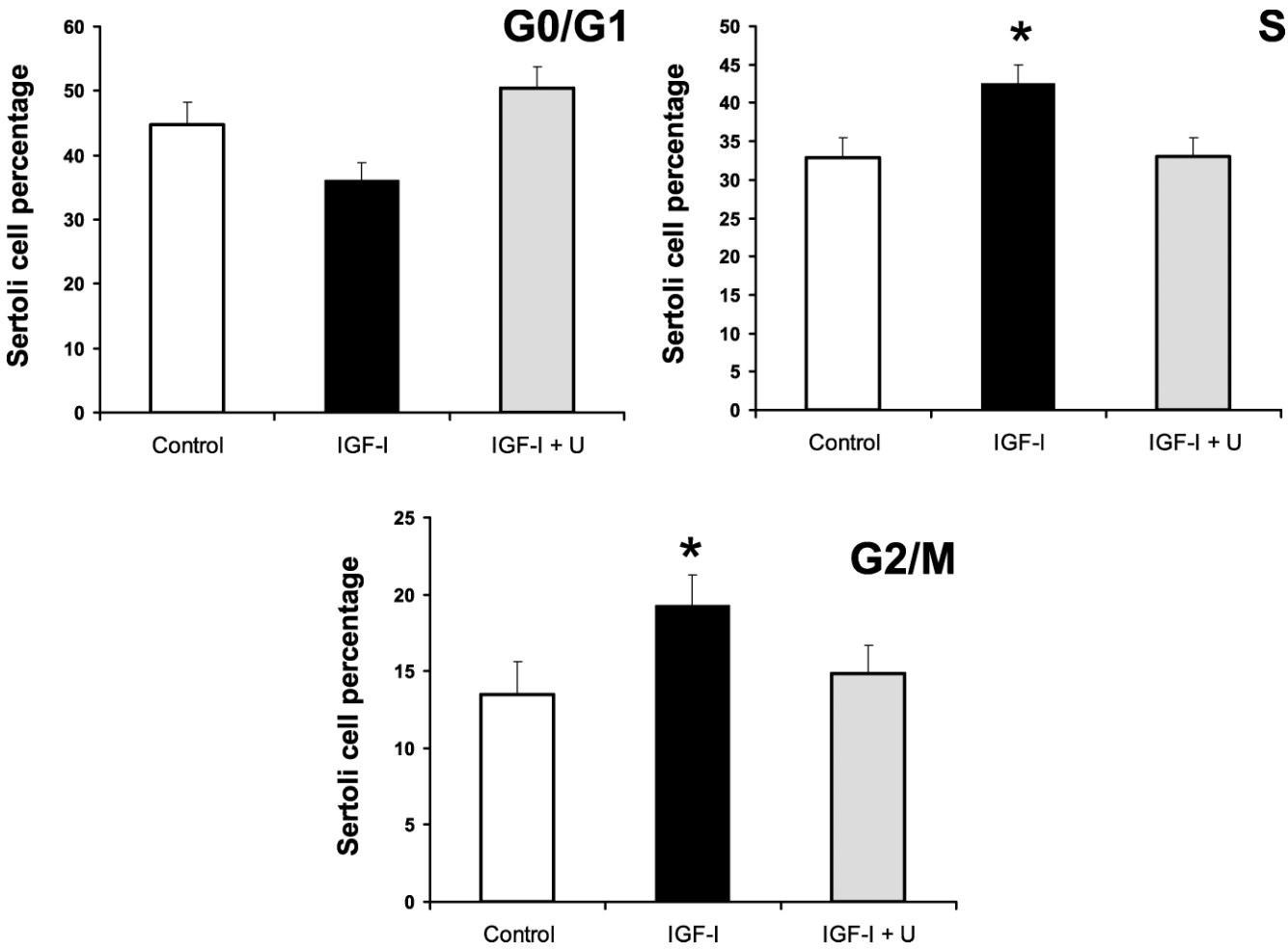

Figure 7. IGF-1 effect on the cell cycle of Sertoli cells analyzed by flow cytometry. Treating cells with IGF-1 diminished the percentage of cells in the G0/G1 phase of the cell cycle, thus promoting a concomitant increase in the percentage of cells in either the S or G2/M phases. Inhibition of the MEK/ERK 1/2 pathway with the UO126 inhibitor reversed the IGF-1 effect. In the S phase, $^*P < 0.02$ for IGF-1 treatment with respect to the control group. In the G2/M phase, $^*P < 0.02$ for IGF-1 with respect to the control group.

Discussion

We demonstrated for the first time *Igf-1* and *Igf-1r* gene and protein expression in the embryonic mouse testis. These results are consistent with those from previous studies in which it was found, through use of RT-PCR, that transcripts for the *Igf-1* gene are expressed in the urogenital ridge at 10.5 days of gestation in the entire testis (8). The IGF-1 protein appears to be expressed to a lesser extent than its mRNA in the male gonad at the onset of testicular development at E12. Measurement of endogenous IGF-1 production by radioimmunoassay experiments revealed that embryonic testes produce small amounts of IGF-1 at E14–E18 (11–19 pg/ml). It may be possible that the small amount of endogenous IGF-1 produced in the mouse testis at this stage is used to regulate other important functions, such as testosterone production. It has been described that endocrine activity of the male gonad starts at 12 days of gestation in the mouse (8).

It was interesting to find that Sertoli cells still have the capacity to proliferate at major stages of testis growth at the fetal (E16) and perinatal (E18) periods. These findings agree with those from previous reports which describe that the major peak of Sertoli proliferation is at 18 days of gestation in the rat (30). Our results demonstrate that proliferation of embryonic somatic cells is coordinated by IGF-1. It is likely that IGF-1 activates D-type cyclins or other growth factors that regulate testis growth. The activation of distinct D-type cyclins has been described in the mouse embryonic gonad (19, 20).

On the other hand, the dynamics of Leydig cell proliferation show that exogenous IGF-1 promotes proliferation from E14–E18. The high percentage of proliferating Leydig cells found with IGF-1 could be related to fetal testis being required to synthesize more steroids to maintain the male phenotype (1). The data presented here are in opposition to findings from previous investigations showing no effect of IGF-1 on Leydig cell proliferation at 16.5 and 18.5 days in the rat embryonic testis (25). These differences in Leydig cell proliferation may be explained by the lower dose of IGF-1 (50 ng) used in the previous study. We found

that 50 ng of IGF-1 had no effect on Leydig or Sertoli cell proliferation. However, differences between rodent species may also be responsible for the observed effects of IGF-1 in rat and mouse testes. The finding that both *Igf-1* and *Igf-1r* are simultaneously expressed in Sertoli and Leydig cells suggests that mouse fetal testis is autocrinally regulated. The MEK/ERK 1/2 inhibitor diminished proliferation of Sertoli and Leydig cells, thus suggesting that proliferation is regulated through the mitogen-activated protein (MAP)-kinase signaling pathway. We found that IGF-1 was located in the nucleus of the somatic testicular cells (unpublished data). Location of IGF-1 in the nucleus suggests that this growth factor probably activates other proteins required for testicular function, as has been observed in the nucleus of the hepatocyte (31). We observed IGF-I strong staining in the nucleus of Leydig cells, whereas in Sertoli cells, this pattern was absent. It is likely that the presence of IGF-1 in the nucleus of Leydig cells may be related to the state of differentiation of the cell. Experiments performed in the embryonic chicken lens demonstrated that IGF-I accumulates in the nuclei of epithelial cells, but not in the fiber cells (32). These findings suggest that transport of IGF-I to different subcellular compartments may be related to the state of differentiation of the cells.

Furthermore, interesting experiments performed in opossum kidney cells demonstrated that both IGF-I and the IGF-I binding protein 3 (IGFBP3) are internalized to the endosome compartment in resting cells. In contrast, in proliferating cells, IGF-I and IGFBP3 accumulated in the nucleus (33).

We found that IGF-Ir was located in the nucleus of Leydig cells. This finding suggests that the IGF-1 receptor could be transported to the nuclear compartment. At the present time, there is no evidence to support this observation. However, experiments performed in various cell lines and IGF-I target cells demonstrated that the IGF-I receptor is necessary to translocate the insulin receptor substrates (IRS) 1–3 to the nucleus. Mutation of the tyrosine kinase domains on these proteins abolishes their translocation to the nucleus (34). An IGF-I receptor subunit was found in the nucleus of Leydig cells. It is possible that the IGF-Ir and the IRS share some homology (34). However, our antibodies were specific to detect the receptors described here. It is thus likely that the immunoreaction observed in the nucleus of this cell type can be due to endogenous IGF-I activating its signaling pathway, leading to activation of the IGF-I receptor, which in turn is used for nuclear translocation of IRS.

Our results demonstrate the importance of IGF-1 expression for growth and function of postnatal testes. *Igf-1* knockout mice are infertile dwarfs that sustained spermatogenesis by, on average, 17%–18%, and their testes were smaller, but less than what was expected, compared with their wild type litter mates (3). These findings indicate that *Igf-I* knockout testis was fine, suggesting a posterior compensation by other factors.

We rule out that IGF-1 is induced *in vitro,* since we tested IGF-I expression in isolated cells before starting and after one, two, or three days of culture, and observed that IGF-I expression was maintained (unpublished data).

We have previously demonstrated that transcripts for *Igf-1* are expressed since the beginning of testicular development at 12–18 days of gestation and that their expression increased according to age (8). *Igf-1* transcripts are also found in isolated cells tested after one, two, or three days of culture (unpublished data). Therefore, it is reasonable to think that IGF-I is naturally expressed.

Previous data obtained by our group (8) and the present results demonstrate that IGF-1 plays a dual role during testis development, first as an enhancer of steroid hormone production (8), and second, as regulator of Leydig and Sertoli cell proliferation in the mouse prenatal testis. Similar functions of IGF-1 have been shown in the testis of adult mammals (11). Proliferation percentage observed in the Sertoli and Leydig cells along testicular development could be related to the receptor number present at each developmental stage. The available receptor number for IGF-1 appears to vary according study tissue (35). Finally, we propose that IGF-1 regulates proliferation autocrinally, through an MAPkinase-signaling pathway, leading to the differentiation and growth of mouse testis.

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