Insulin-Like Growth Factor-I Is an Important Antiapoptotic Factor for Rat Leydig Cells during Postnatal Development

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The present investigation examines the influence of IGF-I and the role of IGF-I receptor (IGF-IR) in the apoptosis/survival of Leydig cells. Immunohistochemical analysis of the rat testis at different ages revealed that the level of the phosphorylated IGF-IR increases from birth to d 20 of postnatal life, remaining high in the adult testis. Western blotting revealed that this level is higher in Leydig cells isolated from 40-d-old than from 10- or 60-d-old rats. Application of the terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay revealed that IGF-I decreases the level of apoptosis in Leydig cells at all stages of development, and the selective inhibitor of IGF-IR, picropodophyllin, blocks this antiapoptotic effect. The mechanism underlying the antiapoptotic action of IGF-I involves the phosphatidylinositol 3-kinase/Akt pathway, and in immature Leydig cells, this growth factor enhances the expression of Bel-2 and cellular inhibitor of apoptosis proteins 2, while preventing activation of caspase-3 by cleavage. Furthermore, IGF-II and high concentrations of insulin also evoke phosphorylation of IGF-IR and, like IGF-I, enhance the expression of the steroidogenic acute regulatory protein by Leydig cells. Inhibition of IGF-IR by picropodophyllin decreases the survival of Leydig cells, both in the presence and absence of IGF-I, demonstrating that signaling via the IGF-IR plays an important role in Leydig cell survival. (Endocrinology 148: 128–139, 2007)

Although gonadal function is under the general control of the hypothalamic-pituitary-gonadal axis, the local actions of gonadotropins are modulated by hormones from other sources as well as by additional factors (1). In the case of the testis, IGF-I within the gonads is one such important factor involved in Leydig cell differentiation, mitogenesis, and steroidogenesis (2, 3). Systemic levels of IGF-I, which can act either as a systemic hormone or local growth factor, increase primarily in response to GH and various nutritional factors (4). The physiological effects of IGF-I are mediated through activation of its specific receptor (IGF-IR), which is also activated by IGF-II and by high concentrations of insulin itself (5, 6). The system consisting of IGF-I and IGF-II, the corresponding receptors (IGF-IR and IGF-IIIR) and IGF-binding proteins, is an important regulator of cell growth, apoptosis, and mitogenesis (6).

IGF-IR, a transmembrane protein exhibiting tyrosine kinase activity, is a tetramer composed of two extracellular α-subunits and two β-subunits each containing a transmembrane domain, an intracellular enzymatically active domain, and a C-terminal domain (7). Upon activation, IGF-IR stimulates downstream signaling mechanisms, including the MAPK pathway associated with growth and proliferation and the phosphatidylinositol 3-kinase (PI3K) pathway associated with inhibition of apoptosis (6, 8).

Although IGF-I acts as an antiapoptotic factor in various tissues (4), little is yet known concerning the direct effects of this hormone on apoptosis in the testis, the occurrence of which is suggested by the expression of IGF-IR in Leydig, Sertoli, and peritubular cells (9). In vitro IGF-I stimulates DNA synthesis and cell proliferation by Sertoli cells isolated from the pig testis (10). In addition, in this same species, IGF-I promotes the differentiation of fetal Leydig cells (11). However, no definitive information concerning the possible effect of IGF-I on Leydig cell survival is presently available.

Certain investigations have focused on the regulation of the programmed death of testicular Leydig cells. For example, corticosterone administered exogenously induces apoptosis in rat Leydig cells, thereby reducing the numbers of these cells in the testicular interstitium (12). Similarly, nicotine promotes apoptosis in Leydig cells, a phenomenon suggested to contributing to subfertility in male smokers (13). Moreover, withdrawal of testosterone results in apoptotic germ cell death in most stages of the cycle of the seminiferous epithelium (14).

Recently, the cyclolignan picropodophyllin (PPP) has been reported to affect the PI3K pathway and induce apoptosis in cells that express IGF-IR (8). In contrast, no such responses were observed in cells lacking this receptor, indicating that PPP does not influence the PI3K pathway by co-inhibiting...
receptors for other growth factors or by interfering directly with Akt and/or any of its downstream events, thus verifying that this effect is mediated specifically by IGF-IR (8).

Two central classes of proteins involved in the cascade leading to apoptotic cell death are members of the Bcl-2 family (15) and a class of cysteine proteases known as caspases (16). The Bcl-2 family consists of two functionally distinct groups of proteins, i.e. antiapoptotic and proapoptotic proteins. Proteins belonging to the Bcl-2 family form homodimers as well as complex combinations of heterodimers with Bax (17) so that the ratio of Bcl-2 to Bax present is an important determinant of the response to apoptotic stimulation (15, 17). IGF-I prevents cytokine-induced alterations in the levels of expression of the members of the Bcl-2 family, counteracting the proapoptotic reduction in Bcl-2 and elevation in Bax caused by cytokines (18, 19).

Although IGF-I is known to inhibit apoptosis in a number of different types of cells, including hematopoietic cells and cells in preovulatory follicles and the mammary gland (4), a corresponding effect on Leydig cells has yet to be demonstrated. Therefore, the present investigation was designed to clarify the effects of IGF-I and the role of IGF-IR activation in connection with Leydig cell apoptosis.

Materials and Methods

Reagents

DMEM/Ham’s nutrient mixture F-12, MEM, Hank’s balanced salts solution without Ca²⁺ or Mg²⁺, and penicillin and streptomycin (Invitrogen Life Technologies, Inc., Paisley, UK) and BSA (fraction V), Percoll, HEPES, and collagenase type I (Sigma-Aldrich, St. Louis, MO) were used to isolate Leydig cells, which were subsequently treated with recombinant rat IGF-I, IGF-II, or GH (Growth Hormone Limited, Adelaide, Australia) or with insulin (Actrapid; Novo Nordisk A/S, Tarska, Denmark). The cell proliferation reagent WST-1 (Roche Diagnostics, Penzberg, Germany), used to determine cell viability, and the Analysis of DNA Fragmentation by Cell Death Detection ELISA® plus kit (Roche Diagnostics, Meylan, France) and TdT-FragEL DNA Fragmentation Detection kit (Calbiochem, Bad Soden, Germany), employed to monitor apoptosis, were purchased from the sources indicated.

The following antibodies were used to perform Western blotting: mouse antiserum ED2 IgG (catalog no. MCA 342R; Serotec, Kidlington, UK); anti-Bcl-2 (Santa Cruz Biotechnology), the phospho-IGF-IR (pIGF-IR) (Tyr1131) (Cell Signaling Technology, Inc., Beverly, MA), pAkt1 (Ser473), Akt1 and Bcl-2 (Santa Cruz Biotechnology), the phospho-IGF-IR (pIGF-IR) (Tyr1131) (Cell Signaling Technology, Inc., Beverly, MA), pAkt1 (Ser473), Akt1 and Bcl-2 (Santa Cruz Biotechnology), the phospho-IGF-IR (pIGF-IR) (Tyr1131) (Cell Signaling Technology, Inc., Beverly, MA), pAkt1 (Ser473), Akt1 and Bcl-2, the phospho-Akt (pAkt) (Ser473), Akt1 and Bcl-2, and pAkt1 (Ser473) (Cell Signaling Technology, Inc., Beverly, MA) (9). The specificity of the IGF-IR antibody was confirmed by preincubation before use with the peptide toward which this antibody was raised as well as by incubation of parallel sections with nonimmune rabbit IgG at the same dilution (negative control). These sections were then counterstained with Mayer’s hematoxylin, dehydrated, and mounted under a coverslip using resin (Pernmount, SP15-100; Fisher Scientific Co., Pittsburgh, PA). Testicular sections from rats with leukemia used as a positive control were immunostained for pIGF-IR (Tyr1136) in an identical manner (20, 21).

Finally, 20 separated fields in each of three nonadjacent sections from each testis were examined for staining employing a Nikon Eclipse E800 microscope (×40 magnification; Nikon, Inc., Melville, NY) equipped with a SPOT RT digital camera (Diagnostic Instruments, Inc., Nikon) interfaced with a computer.

Isolation and culture of Leydig cells

Leydig cells from rats older than 40 d were prepared according to the procedure of Klinefelter et al. (22) as described earlier (23, 24). The corresponding cells from 10-d-old rats were obtained as described by Khan et al. (25). The purity of the Leydig cell preparations was more than 90%, as determined by histochemical staining of this cell type for 3β-hydroxysteroid dehydrogenase (26). Exclusion of trypsin blue demonstrated that the cell viability was routinely greater than 96%.

Subsequently, 1.5 × 10⁶ cells in a total volume of 200 μl were routinely plated in individual culture wells on 96-well plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 h. At this point, the culture medium was replaced by fresh medium containing IGF-I (0.1–100 ng/ml) and in additional experiments IGF-I (500 ng/ml), PPP (100 nmol/liter), and/or PD98059 (50 μM) and then incubated for an additional 24 h. In the case of Western blotting, 2 × 10⁶ cells were incubated on six-well plates (Falcon) for 24 h, after which the culture medium was replaced by fresh medium with or without IGF-I (10 ng/ml), GH (10 ng/ml), IGF-II (10 ng/ml), insulin (1000 ng/ml), and/or PPP (1–1000 nmol/liter) and incubated for an additional 24 h.

DNA synthesis by Leydig cells in vitro

After treatment as described above, Leydig cells from 40-d-old rats on 96-well plates were labeled for the final 4 h of culture with 1[³H]thymidine (1 μCi per well; Amersham Pharmacia Biotech, Little Chalfont, UK), and the radioactivity incorporated (cpm) subsequently determined using a Beckman scintillation spectrometer. In each individual experiment, triplicate or quadruplicate cell cultures were exposed to each treatment and four independent experiments were performed.

Determination of the number of viable Leydig cells in cultures

The numbers of viable Leydig cells in culture were determined by the WST-1 procedure (Roche Diagnostics GmbH, Mannheim, Germany), which is based on the fact that active mitochondria hydrolyze the tetrazolium salt WST-1 to produce a soluble, colored formazan salt that can be quantitated spectrophotometrically.

Impairment of the number of viable Leydig cells in culture

The numbers of viable Leydig cells in culture were determined by the WST-1 procedure (Roche Diagnostics GmbH, Mannheim, Germany), which is based on the fact that active mitochondria hydrolyze the tetrazolium salt WST-1 to produce a soluble, colored formazan salt that can be quantitated spectrophotometrically. Thus, this conversion occurs only in viable cells and reflects the number of such cells present. Accordingly, after treatment of Leydig cells in culture with IGF-I (0.1–100 ng/ml), 1–1000 nmol/liter PPP, 10 nmwortmannin, and/or 50 μM
PD98059 as described above, 100 μl serum-free DMEM and 10 μl WST-1 were added to each well and the increase in absorption at 450 nm monitored for 60 min. PPP or Wortmannin was added to the medium 1 h before the addition of IGF-I.

Determination of Leydig cell apoptosis in vitro: detection of cell death

The extent of apoptosis occurring in the cultured Leydig cells was assessed with an ELISA kit designed for both qualitative and quantitative photometric determination of cytoplasmic levels of histone-associated DNA fragments. In this case, Leydig cells (1.5 x 10^5 per well) were cultured on 96-well plates for 24 h, after which these cells were pre-treated with 100 nmol PPP, 10 nm wortmannin, 50 μM PD98059, and/or different concentrations of IGF-I (0.1–100 ng/ml) for an additional 24 h. Thereafter, attached cells were harvested and centrifuged at 1500 rpm for 6 min, after which the resulting pellets were treated in accordance with the manufacturer’s instructions. The increase in the level of nucleosomes present in the cytoplasm of treated cells was calculated by comparison with untreated cells and these levels then expressed as percentages of the corresponding control value.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining

After isolation of Leydig cells and treatment with 10 ng IGF-I/ml or 100 nmol PPP as described above, fragmented DNA was detected using the TUNEL procedure (TdT-FragEL DNA fragmentation detection kit, catalog no. QIA33; Calbiochem, Germany) in accordance with the manufacturer’s instructions. Briefly, after culture of Leydig cells on coverslips (Beckton Dickinson) placed in 24-well plates, the cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4 C and subsequently washed twice with PBS. Next, the cells were permeabilized with proteinase K for 1 h at room temperature, washed with PBS, and thereafter incubated with streptavidin/Alexa Fluor 546 conjugate ( Molecular Probes, Inc., Eugene, OR) for 1 h at 37 C in the dark. The cells were washed twice with PBS and mounted in Vectashield Hard Set (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) for 1 h at room temperature in the dark to stain all nuclei.

The dual-fluorescence emission from the Leydig cells involving both apoptotic (Alexa, red) and nuclear signals (DAPI, blue) was detected using an Eclipse E800 microscope (Nikon).

To obtain an accurate estimate of the overall apoptotic incidence, digital images of the stained sections were acquired and processed with the Image Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD, USA) and, after additional washing, detected employing donkey antirabbit or sheep antimouse IgG secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Finally, for detection, these blots were incubated with ECL Plus Western blotting agent and then exposed to ECL Hyperfilm (Amersham Pharmacia Biotech).

The total proteins level and the level of phosphorylated forms were assessed using membranes processed in an identical fashion. Detection of bound antibody by enhanced chemiluminescence and stripping and reanalysis of the membranes was also accomplished in accordance with the manufacturer’s instructions.

Determination of androgen production

Media collected from the various cell cultures described above were stored at −20 C before assaying for testosterone and 5α-androstan-3α,17β-diol, the predominant androgens synthesized by immature Leydig cells. Testosterone was quantitated employing a Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA), in accordance with the manufacturer’s instructions. The cross-reactivity of 5α-androstan-3α,17β-diol in the testosterone RIA was 0.4%. The level of 5α-androstan-3α,17β-diol was determined by RIA using specific antiserum (Cosmo Bio Co. Ltd., Tokyo, Japan) and radiolabeled 5α-[9,11-3H] (N)androstan-3α,17β-diol (specific activity = 40 Ci/mmol; NEN Life Science Products, Boston, MA). The cross-reactivity of testosterone in this RIA was 0.2%.

Statistical analyses

All results are presented as mean values ± sd. Comparison of the androgen levels (i.e. the sum of the levels of 5α-androstan-3α,17β-diol and testosterone) was performed using a one-way repeated-measures ANOVA, together with Tukey’s post hoc test for pairwise comparisons.

Rates of cell proliferation, cell survival, Western blot, and cell death detection were compared using one-way repeated-measures ANOVA, employing the HOLM-Sidak procedure for all pairwise multiple comparisons. In all cases, a P value of <0.05 was considered to be statistically significant.

Results

Pattern of expression and cellular distribution of pIGF-IR during postnatal testicular development

Immunohistochemical analysis of the testis of rats at different ages revealed that activation (phosphorylation) of IGF-IR increased dramatically from 20 d after birth (Fig. 1, A–D), with an even higher level of pIGF-IR being observed in the adult testis (Fig. 1, G–J). On postnatal d 10, Leydig cells, germ cells, and peritubular cells stained positively for pIGF-IR (Fig. 1A). On postnatal d 20, Leydig cells and pachytene spermatocytes stained positively for pIGF-IR (Fig. 1C), whereas at 60 d of age, Sertoli cells, spermatogonia, spermatids, and Leydig cells were also stained (Fig. 1, G–K). These findings were supported further by Western blotting (Fig 2A), which demonstrated that the level of pIGF-IR was 2.4- to 5.3-fold higher in 40- and 60-d-old rat testes than in the 10-d-old testis. Testis from 60-d-old rats infiltrated with leukemia cells revealed high activation (phosphorylation) of IGF-IR (Fig. 1, L and M). Moreover, Leydig cells isolated from 40-d-old rats exhibited higher levels of pIGF-IR (2.0-fold) than did Leydig cells from either 10- or 60-d-old rats (Fig. 2B).

Influence of IGF-I on the DNA synthesis, survival, and apoptosis of rat Leydig cells in culture

The effect of IGF-I on the proliferation of cultured Leydig cells isolated from 10-d-old rats was measured. Untreated immature Leydig cells showed a spontaneous decline in DNA synthesis over 48 h in culture (1200 ± 100 cpm, mean ± sd, for harvest at 4 h vs. 435 ± 132 cpm for harvest at 48 h). Higher concentrations of IGF-I stimulated the rate of DNA synthesis at 48 h in comparison with the initial rate of DNA synthesis (10 ng/ml IGF-I, 555 ± 145 cpm; 100 ng/ml IGF-I,
1310 ± 200 cpm; 500 ng/ml IGF-I, 4628 ± 345 cpm). In 40-d-old rats, proliferation, viability, and apoptosis of cultured Leydig cells were examined. IGF-I (0.1–500 ng/ml) enhanced the rate of DNA synthesis by these cells (as assessed on the basis of the incorporation of [3H]thymidine into DNA between 44 and 48 h of incubation) in comparison with untreated cells. However, no such enhancement was observed in comparison with the initial rate of DNA synthesis at the onset of culture (0–4 h of incubation, Fig. 3A), indicating that IGF-I exerts a maintenance or survival effect rather than a mitogenic action. In contrast to 10-d-old Leydig cells, the pharmacological concentration of 500 ng/ml IGF-I failed to stimulate DNA synthesis of 40-d-old Leydig cells (data not shown).

Assessment of the viability of cultures of Leydig cells isolated from 40-d-old rats by the WST-1 procedure mirrored these findings concerning incorporation of [3H]thymidine (Fig. 3B). Thus, without treatment, the number of viable cells remaining after 48 h of incubation was 28% lower than at the beginning of the culture period, but IGF-I was able to prevent this loss (Fig. 3B). The viability of cells in the different experimental groups is presented in Fig. 3B. PPP inhibited this
survival effect of IGF-I, and furthermore, this compound resulted in an elevated level of apoptosis, whereas IGF-I attenuated apoptosis (Fig. 3C).

These observations were further confirmed using the TUNEL procedure. Accordingly, Leydig cells exposed to PPP (Fig. 4A, I, IV, and VII) displayed a higher rate of apoptosis than untreated cells at all stages of maturation, whereas treatment with IGF-I significantly reduced apoptosis in Leydig cells (Fig. 4A, III, VI, and IX; \( P < 0.01 \)). Representative results for TUNEL staining of Leydig cells from 10-, 40-, and 60-d-old rats are presented and summarized in Fig. 4, A and B.

Next, we employed immunohistochemistry to reveal that Bcl-2 is expressed strongly by Leydig cells isolated from the
testis of 40- and 60-d-old rats (Fig. 5I, A–F). This expression was enhanced by IGF-I (Fig. 5II) and blocked by PPP (not shown). Subsequently, we found that exposure to PPP resulted in the cleavage of caspase-3 into active fragments (20 and 17 kDa), whereas IGF-I completely blocked caspase-3 activation (Fig. 5III).

Mechanisms by which IGF-I promotes the survival of isolated rat Leydig cells

In an attempt to elucidate the mechanism by which IGF-I promotes the survival of isolated rat Leydig cells, the effects of PPP (an inhibitor of IGF-IR), wortmannin (an inhibitor of the PI3K/Akt pathway), and PD98059 (an inhibitor of the MAPK pathway) on this phenomenon were examined. As shown in Fig. 6, PPP significantly decreased the rate of DNA synthesis by IGF-I-stimulated Leydig cells (Fig. 6A), and both PPP and wortmannin abolished the enhancement in survival caused by IGF-I (Fig. 6B), whereas PD98059 had no such effect (Fig. 6 B). Assay of cell death indicated that IGF-I decreased the level of apoptosis in Leydig cells by 55% compared with untreated control cells and that wortmannin blocked this effect, whereas PD98059 did not (Fig. 6C).

Stimulation of the phosphorylation of IGF-IR by various hormones

As expected, treatment of isolated rat Leydig cells with an IGF-I concentration of 10 ng/ml stimulated phosphorylation of IGF-IR, and this effect was maximal after 5 min (Fig. 7I, A).
Interestingly, exposure to the same concentration of IGF-II resulted in a similar pattern of IGF-IR phosphorylation (Fig. 7I, B), indicating a similar affinity for IGF-IR. High levels of insulin (1000 ng/ml) also resulted in a phosphorylation of this receptor, with a maximum after 15 min (i.e., 10 min later than in the case of IGF-I), suggesting that insulin binds to the...
same receptor but with a lower affinity than IGF-I (Fig. 7I, C). In contrast, GH (10 ng/ml) had no effect on phosphorylation of IGF-IR (Fig. 7I, D).

Site of action of PPP

IGF-I (10 ng/ml) stimulated phosphorylation of IGF-IR (Tyr1131) (Fig. 7II, A). Investigation of the site of action of PPP employing two different antibodies against pIGF-IR, specific for phosphorylation at Tyr1131 and Tyr1136, respectively, revealed that PPP reduced the level of phosphorylation at both these sites (Fig. 7II, B and C).

Mechanism of the antiapoptotic action of IGF-I

In attempt to elucidate the antiapoptotic mechanism(s) activated by IGF-I, this hormone was found to promote phosphorylation and, thus, activation of Akt in isolated Leydig cells, an effect that was maximal after 10 min (Fig. 8I, A) and could be significantly attenuated by PPP (Fig. 8I, B). These observations suggest that the PI3K pathway, which is critical for cell survival, might be activated in connection with IGF-I signaling. Indeed, treatment of Leydig cells with wortmannin, a selective inhibitor of PI3K, dramatically suppressed the level of pAkt in IGF-I-stimulated Leydig cells (Fig. 8I, C); wortmannin alone had a similar effect as PPP alone (not shown). Moreover, the expression of c-IAP2 by Leydig cells was enhanced by IGF-I (Fig. 8I, D).

Role of IGF-IR in Leydig cells steroidogenesis

Because the primary function of Leydig cells is production of androgens, we examined the influence of IGF-I, IGF-II, GH, and insulin on steroidogenesis and the expression of StAR by these cells. All four of these hormones up-regulate the expression of STAR (Fig. 8II, A), and in all cases this effect could be significantly attenuated by blocking IGF-IR with PPP (Fig. 8II, A). A similar pattern was found with respect to the production of androgens by the isolated rat Leydig cells (Fig. 8II, B). Interestingly, stimulation of StAR expression and androgen production by GH were blocked by PPP to a lesser extent than in the case of other hormones, indicating that GH may also up-regulate the expression of StAR directly. The predominant androgen produced by 40-d-old Leydig cells was 5α-androstane-3α,17β-diol. The concentration of this steroid was 4:1 in relation to testosterone.

Discussion

The major objectives of this study were to investigate the effects of IGF-I on the survival, apoptosis, and phosphorylation of IGF-IR and expression of Bcl-2 and c-IAP2 by rat Leydig cells, including the possible link between this paracrine factor and the antiapoptotic PI3K/Akt pathway and the inhibitory action of PPP. IGF-I is involved in protecting fibroblasts, motoneurons, hematopoietic cells, chondrocytes, and other types of cells against apoptosis (4). Cell survival mediated via this receptor involves activation of PI3K and MAPK pathways (27, 28), and the resulting antiapoptotic effect depends on the cell type being examined.

Growth factors, e.g. IGF-I, can activate one or both of these pathways (5, 6). The relative importance of the MAPK and...
PI3K pathways in protecting various types of cells against apoptosis has been examined employing selective inhibitors. In certain cells, both pathways protect against apoptosis, whereas in others, one or the other is used exclusively (28).

Recently, the MAPK pathway has been found to be primarily involved in regulating steroidogenesis in the case of Leydig cells (29).

The present investigation demonstrated for the first time that IGF-I also protects Leydig cells against apoptosis. Because a link between IGF-I and the PI3K/Akt pathway has been proposed to be involved in the prevention of apoptosis in other types of cells (30), we examined Leydig cell phosphorylation of Akt and IGF-IR in response to IGF-I in the absence and presence of PPP or inhibitors of the PI3K or MAPK pathway. When the level of survival is high, IGF-IR (Tyr1136) and Akt are phosphorylated, whereas when survival is low, the corresponding levels of phosphorylation are low or undetectable. Indeed, the level of survival is correlated with the degree of Akt phosphorylation. Thus, Akt appears to be a key signaling factor in preventing Leydig cell apoptosis.

Activation of Akt is known to be of general importance in connection with the prevention of apoptosis in mammalian cells, because this kinase phosphorylates and inactivates caspase-9 and Bad (15, 18, 31). Furthermore, Akt activates nuclear factor-κB (a family of transcription factors charac-

![Fig. 7. I, Effects of IGF-I (A), IGF-II (B), insulin (C), and GH (D) on phosphorylation of IGF-IR at Tyr1136. Leydig cells (2 × 10^6 per well) were cultured and treated as described in Materials and Methods. Subsequently, cellular protein was extracted and subjected to Western blotting using an antibody toward pIGF-IR (Tyr1136) and, after stripping the membrane, blotted again with polyclonal antibodies against the β-subunit of IGF-IR (C-20) and α-tubulin as internal control for constant loading. The OD values are the means ± SD of the densitometric values obtained in three independent experiments, and above each OD, a representative Western blot is shown. a, P < 0.05 compared with the initial value (i.e., at time 0). II, Effects of PPP on IGF-I-stimulated phosphorylation of IGF-IR at Tyr1131 and Tyr1136. Leydig cells (2 × 10^6 per well) were cultured and treated as described in Materials and Methods. Subsequently, cellular protein was extracted and subjected to Western blotting using antibodies specific for IGF-I phosphorylated at Tyr1131 and Tyr1136. The effects of IGF-I on phosphorylation of IGF-IR at Tyr1131 (A), inhibition of IGF-I-stimulated phosphorylation of IGF-IR at Tyr1131 by PPP (B), and inhibition of IGF-I-stimulated phosphorylation of IGF-IR at Tyr1136 by PPP (C). The OD values are the means ± SD of the densitometric values obtained in three independent experiments, and above each OD, a representative Western blot is shown. a, P < 0.05 compared with the initial value (i.e., at time 0); b, P < 0.05 compared with cells treated with IGF-I.](https://academic.oup.com/endo/article-abstract/148/1/128/2501014)
IGF-I Survival Factor for Leydig Cells

Fig. 8. I, Effects of IGF-I on the phosphorylation of Akt and expression of c-IAP2 in Leydig cells and inhibition of the former effect by PPP and wortmannin. Leydig cells (2 × 10⁶ per well) were cultured and treated as described in Materials and Methods. Subsequently, cellular protein was extracted and subjected to Western blotting using antibodies directed toward pAkt or all forms of this protein (A–C) or toward c-IAP2 and, after stripping, α-tubulin as an internal control for constant loading (D). A, Time course of IGF-I-stimulated phosphorylation of Akt; B, inhibition of IGF-I-stimulated phosphorylation of Akt by PPP; C, inhibition of IGF-I-stimulated phosphorylation of Akt by 100 nM PPP or 10 nM wortmannin; D, time course of the increased expression of c-IAP2 stimulated by IGF-I. The OD values are the means ± SD of the densitometric values obtained in three independent experiments, and above each OD, a representative Western blot is shown. a, P < 0.05 compared with the initial value (i.e., at time 0); b, P < 0.05 compared with cells treated with IGF-I, II, IGF-I, IGF-II, GH, and insulin (INS) all up-regulated the expression of StAR and stimulated production of androgens by isolated rat Leydig cells, and these effects can be attenuated by blocking IGF-IR with PPP. A, Leydig cells isolated from 40-d-old rats (2 × 10⁶ per well) were cultured and treated as described in Materials and Methods. Subsequently, cellular protein was extracted and subjected to Western blotting using antibodies directed toward StAR and, after stripping, α-tubulin as an internal control for constant loading. A representative Western blot is depicted here. OD values are the means ± SD of the densitometric values obtained in three independent experiments performed as in A. a, P < 0.05 compared with the corresponding value for cells treated with hormone alone. B, In this case, 7.5 × 10⁴ cells per well were treated as in A, after which the culture media were collected for determination of total androgen levels (the sum of the levels of testosterone and 5α-androstan-3α,17β-diol). The means ± SD for three independent experiments (each involving triplicate determinations) are shown. a, P < 0.05 compared with the corresponding value for cells treated with hormone alone.

Our findings also clearly reveal that Bcl-2 is expressed by Leydig cells and that this expression can be enhanced by IGF-I, suggesting that this phenomenon may explain, at least in part, the antiapoptotic influence of IGF-I in Leydig cells. A recent study reports that the increased frequency of apoptosis in Leydig cells exposed to nicotine is associated with down-regulation of the expression of Bcl-2, thus corroborating the importance of this protein in this context (13). The antiapoptotic activity of Bcl-2 includes, among other things, antioxidant activity, which preserves the mitochondrial membrane potential and blocks the release of cytochrome c,
thereby preventing activation of the caspases (34). Indeed, isolated rat Leydig cells treated with IGF-I exhibit reduced levels of activated caspase-3, further indicating that this event may be involved in the paracrine and autocrine stimulation of DNA synthesis and survival of Leydig cells by IGF-I, at least in vitro.

The antiapoptotic effect of IGF-I may be of importance for steroidogenesis and spermatogenesis. An interesting study indicates that IGF-I levels are higher in seminal plasma of fertile men compared with infertile men. The levels of IGF-I were linearly correlated with the total sperm counts in the semen, possibly reflecting an augmenting role in spermatogenesis (35).

The positive effect of IGF-I on Leydig cell survival is abolished by PPP, which reduces Akt phosphorylation by inhibiting PI3K and other kinases upstream of Akt. One possible explanation for this finding is that PPP decreases phosphorylation of IGF-IR at two different sites (i.e. Tyr1136 and Tyr1131), thereby blocking signaling to downstream anti-apoptotic pathways, such as that involving PI3K and Akt, and promoting apoptosis. This explanation is supported by previous investigations on the effects of PPP in other model system (8). PPP has been demonstrated to inhibit the IGF-IR activity (36), whereas it did not inhibit activity of the highly homologous insulin receptor (36). Furthermore, PPP does not inhibit phosphorylation of other major growth factor receptors either, such as platelet-derived growth factor receptor, epidermal growth factor receptor, and fibroblast growth factor receptor (36). PPP causes tumor regression, or decreases tumor burden, in IGF-II-expressing tumors (36–38), whereas tumor xenografts made up of IGF-IR-negative v-src-transformed cells were not responsive to PPP (36). Moreover, primary cultures of human craniopharyngioma cells with low or no IGF-I expression exhibited no or only slight growth inhibition upon treatment with PPP (39). Menu et al. (38) showed that proliferation of the multiple myeloma cell line 5T33MM was efficiently blocked by PPP, whereas the 5T33MMvmt cell line, clonally identical to the 5T33MM line but lacking the IGF-IR, was not affected. All these observations indicate a selective action of PPP on the IGF-IR.

LH is a main regulator of Leydig cell development and function (3). However, results from IGF-I null mice showed that mice with a targeted deletion of IGF-I had an abnormal pattern of Leydig cell proliferation and differentiation and decreased androgen production in response to LH. We demonstrated that IGF-I was able to stimulate proliferation in Leydig cells from 10-d-old rats but not in Leydig cells from 40-d-old rats. This result is in accordance with previous in vitro experiments showing that IGF-I stimulates proliferation and differentiation of Leydig cell precursors (40, 41), indicating that IGF-I effects are dependent on the stage of development. However, additional studies on the combination of IGF-I and LH are necessary to clarify the possible collaborative effect of IGF-I and LH on Leydig cell survival and apoptosis.

Interestingly, both IGF-II and insulin (at a high pharmacological concentration) phosphorylate/activate IGF-IR in the same manner as IGF-I, suggesting that all of these factors activate common signaling pathways to produce similar effects on Leydig cells (albeit with a delay in the case of insulin). Certain other reports have proposed that IGF-II plays a role in spermatogenesis and tumor growth (42), and others have demonstrated that IGF-II is expressed by Sertoli, Leydig, and germ cells (42, 43). Thus, IGF-II may participate in regulating the proliferation and apoptosis of Leydig cells, but additional research is required to elucidate the underlying mechanism(s) especially with respect to the prevention of apoptosis.

Because the main primary function of Leydig cells is the production of androgens, we evaluated effects of IGF-I, IGF-II, and insulin on the expression of StAR and rate of steroidogenesis by these cells. The observation that all three of these stimulate both of these processes indicates that these hormones may share a common signaling pathway involving IGF-IR. In contrast, GH does not directly activate IGF-IR in isolated rat Leydig cells but stimulates steroidogenesis and StAR expression most probably using its own receptor.

A previous investigation revealed that one of the mechanisms by which manganese promotes apoptosis in Leydig cells is by decreasing the cytoplasmic level of the StAR protein, which results in alteration of the potential across the mitochondrial membrane (44). The finding is in agreement with our result suggesting that the level of StAR protein is related to the effect of IGF-I on the survival of Leydig cells. Although ERK1/2 participates in the regulation of StAR expression in Leydig cells (45), data indicate that the PI3K pathway plays the major role in connection with this anti-apoptotic effect of IGF-I.

Overexpression of IGF-IR is associated with the proliferation, adhesion, and metastasis of tumor cells (46). In the case of the testis IGF-IR has also been implicated as playing a role in tumor cell proliferation (42), whereas IGF-I is involved in normal spermatogenesis (47). Moreover, previous studies have demonstrated that the levels of expression of IGF-IR and the FSH receptor by Sertoli cells are correlated, although the IGF-IR is expressed constitutively in both testicular tissue and seminoma (42). Interestingly, whereas the expression of IGF-IR is unaltered, expression of FSH receptor in seminoma is down-regulated, resulting in a lack of spermatogenesis. The proposal that IGF-IR may play a role in tumor growth (42) is in agreement with our finding that this receptor is overexpressed by leukemic cells in the testis and suggests that inhibitors such as PPP might prove to be useful chemotherapeutic agents.

In summary, we have demonstrated here, using both testicular tissue and primary Leydig cells isolated from rats of different ages, that IGF-IR is activated in the testis in a developmental manner. This receptor plays an important role in preventing apoptosis and thus promoting the survival of Leydig cells. In addition to IGF-I, the IGF-IR can be activated by IGF-II and high concentrations of insulin. The ability of IGF-I to attenuate apoptosis in Leydig cells and promote survival involves the PI3K/Akt pathway, Bcl-2, and c-IAP2.

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