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Insulin-like growth factor-1 (IGF-1) stimulates the IGF binding protein system in rat theca interstitial cells

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There has been considerable interest in rat ovarian insulin-like growth factor binding proteins IGFBPs because they are potent inhibitors of FSH action. *In situ*, IGFBP-2 and -4 and IGFBP-3 mRNAs are expressed in rat theca interstitial (TIC) and theca lutein cells respectively. Although much is known about IGFBPs in rat TIC at the mRNA level, the synthesis and regulation of IGFBP proteins remain poorly understood. The purpose of this study was to identify the species of IGFBPs produced by TIC and to determine the effects of LH and IGF-1 on their expression. This was accomplished by culturing rat TIC for 2 days in serum-free medium with graded doses of LH and/or IGF-I, and measuring IGFBP mRNAs in the cells and IGFBP proteins in the conditioned media by RT-PCR and Western immunoblotting respectively. The RT PCR analysis identified strong bands for IGFBP-2 and -4 mRNAs in TIC. In some treatments, the mRNAs for IGFBP-3 and -6 were also identified, but transcripts for IGFBP-1 and -5 were undetectable. Two species of IGFBPs were detected in the conditioned media of control (untreated) TIC, the 31 kDa IGFBP-2 and the 24 kDa (non-glycosylated) and 28 kDa (glycosylated) forms of IGFBP-4. There was no detectable IGFBP-5 and barely detectable amounts of IGFBP-3 and -6 in the conditioned media. Treatment with LH (0.2–20 μU/ml) caused no significant changes in the levels of the 31 kDa IGFBP-2 and the 24 kDa and 28 kDa IGFBP-4 bands, and there was no detectable IGFBP protease activity. In contrast, IGF-I (100 ng/ml) stimulated the expression of IGFBP-2, IGFBP-4 and a 17.5 kDa IGFBP-4 fragment. The immunoreactive IGFBP-4 fragment suggests the media contained an IGFBP-4 protease. The IGF-I effects were dose dependent ($ED_{50} = 12.4 \pm 3.3$ ng/ml). Co-treating TIC with LH (0.2–20 μU/ml) caused no significant change in the activity of IGF-I in stimulating the expression of IGFBP-2, IGFBP-4 and IGFBP-4 protease. We have demonstrated that IGF-I acts directly on rat TIC to stimulate the expression of the intrinsic IGFBP system. LH, either alone or together with IGF-I, did not significantly change the expression of TIC IGFBP proteins. Therefore, we hypothesize that IGF-I, but not LH, may be a physiologically important regulator of the IGFBP system in rat TIC. Because IGF-I is a potent stimulator of theca function, changes in the expression of this intrinsic IGFBP system could have new implications for ovarian androgen production, both at the physiologic and pathophysiologic levels.

Keywords: Rat Theca; IGF-I; IGFBPs

Introduction

Theca interstitial cells (TIC) produce progestins and androgens during follicle development (Erickson *et al.*, 1985). LH is the major regulator of steroidogenesis in TIC and the effects of LH are mediated through the cyclic adenosine-monophosphate (cAMP) protein kinase A (PKA) pathway (Erickson, 1993). Activation of the LH receptor signal transduction pathway increases the expression of cholesterol side chain cleavage ($P450_{scc}$), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17 α-hydroxylase C17-20 lyase ($P450_{17\alpha}$) gene transcription and translation, which in turn increases the synthesis and secretion of steroid hormones by TIC (Magoffin & Erickson, 1994).

In addition to LH, insulin-like growth factor I (IGF-I) is a potent modulator of rat TIC responses (Magoffin & Erickson, 1994). The IGF-I effects on TIC are mediated by IGF-I receptors (Cara & Rosenfeld, 1988; Hernandez *et al.*, 1988), presumably through increased protein tyrosine kinase activity. *In vitro* studies with TIC have demonstrated that IGF-I itself can stimulate the expression of $P450_{scc}$ (Magoffin *et al.*, 1990; Magoffin & Weitsman, 1993a) type I 3β-HSD (Magoffin & Weitsman, 1993b,c), and LH receptor (Magoffin & Weitsman, 1994). Furthermore, IGF-I acts synergistically with LH to increase the level of androgen biosynthesis (Cara & Rosenfeld, 1988; Hernandez *et al.*, 1988; Magoffin & Erickson, 1988a). The synergy is caused by increased expression of 3β-HSD (Magoffin & Weitsman, 1993b), $P450_{SCC}$ and $P450_{17\alpha}$ (Magoffin *et al.*, 1990; Magoffin & Weitsman, 1993a,c). These data provide evidence that IGF-I is a physiologically relevent stimulator of TIC steroidogenesis. This stimulatory effect may result from the expression of IGF-I by the granulosa cells (Oliver *et al.*, 1989; Hernandez *et al.*, 1989; Zhou *et al.*, 1991).

The IGF binding proteins (IGFBPs) are key components in the IGF system because they influence IGF bioactivity (Rechler, 1993). There are six different IGFBPs (denoted IGFBP-1, -2, -3, -4, -5 and -6), each showing considerable amino acid sequence homology (Shimasaki & Ling, 1992). *In situ* hybridization experiments in adult rat ovaries have demonstrated that the mRNAs for the six IGFBPs are expressed in a tissue specific manner and that the levels of expression change during the estrous cycle (Erickson *et al.*, 1994a). The regulated production of the IGFBPs is therefore implicated in the functions of the ovary. Indeed, an inhibitory role for IGFBP-2, -3, -4 and -5 in FSH action has been established (Ling *et al.*, 1990; Erickson *et al.*, 1994a).

The mRNAs for IGFBP-2 (Nakatani *et al.*, 1991; Erickson *et al.*, 1994b) and IGFBP-4 (Erickson *et al.*, 1992, 1994a) are expressed in rat TIC during follicle development and transcripts for IGFBP-3 are present in the theca lutein cells (Erickson *et al.*, 1993). The estrous cycle is associated with dramatic changes in the levels of these mRNAs. This observation suggests that hormones and/or growth factors regulate IGFBP gene activity during thecogenesis (Erickson *et al.*, 1994a; Magoffin & Erickson, 1994). Consistent with this hypothesis is the identification of changes in the amount of IGFBP-2 mRNA (Ricciarelli *et al.*, 1991), IGFBP-3 (Ricciarelli *et al.*, 1992), and IGFBP-6 (Rohan *et al.*, 1993) mRNAs in rat TIC following hypophysectomy. Although much is known about the expression of IGFBP mRNAs in TIC, almost nothing is known concerning the expression of

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the IGFBPs at the protein level. To fill this gap in our knowledge, we have used a physiologically relevent *in vitro* model system to investigate the effects of LH and IGF-I on the expression of the IGFBP system in rat TIC.

Results

Effect of IGF-I and LH on IGFBP mRNA

We first determined the kinds of IGFBP mRNAs that were expressed by rat theca-interstitial cells. Figure 1 shows the results of RT-PCR of RNA extracts from cultured TIC using specific primers for IGFBP-1, -2, -3, -4, -5 and -6. IGFBP-1 and IGFBP-5 were not detected in untreated TIC or in cells cultured with LH or IGF-I alone or in combination. In untreated TIC there were strong bands for IGFBP-2 and IGFBP-4. IGFBP-3 was not detectable and there was a very weak IGFBP-6 band. In TIC treated with IGF-I or LH alone, there were strong bands for IGFBP-3, -4 and -6 and a moderate IGFBP-2 band. When the TIC were treated with LH plus IGF-I there was a moderately strong IGFBP-2 band, very strong IGFBP-3 and IGFBP-4 bands, but IGFBP-6 was undetectable. These data demonstrate that rat TIC express mRNA for IGFBP-2, -3, -4 and -6 but not IGFBP-1 or -5 and that the pattern of IGFBP mRNA expression is regulated by IGF-I and LH.

Effects of LH and IGF-I on IGFBP production

When TIC conditioned medium was subjected to Western blotting using specific IGFBP antisera, two species were readily detected, namely IGFBP-2 and -4. There was no detectable IGFBP-5 and barely detectable amounts of IGFBP-3 and -6 in the conditioned media (data not shown). As shown in Figure 2A, control cells secreted detectable amounts of IGFBP-2 which migrated as a single 31 kDa band. The basal levels of IGFBP-2 were increased significantly ($\sim$3.5-fold, $P<0.01$) by IGF-I (30 ng/ml), whereas LH had little or not effect. The stimulatory effect of IGF-I on IGFBP-2 was not changed when TIC were co-treated with 20 μU/ml LH (Figure 2A). The other species was IGFBP-4 which migrated as a major 24 kDa band and a minor 28 kDa form in IGF-I treated (Figure 2B). The conditioned media from control TIC also contained detectable amounts of the 24 kDa IGFBP-4 protein (Figure 2B). Basal levels of IGFBP-4 were significantly increased (2.5-fold, $P<0.01$) by IGF-I, whereas LH was without effect. Interestingly, IGF-I (30 ng/ml) also increased the intensity of a band that migrated at 17.5 kDa. This smaller immunoreactive IGFBP-4 band correlates with the predicted size of the IGFBP-4 fragment formed by the hydrolysis of the 24 kDa band by a specific IGFBP-4 protease (Liu *et al.*, 1993). There was no change in the stimulatory effects of IGF-I on the intensities of the IGFBP-4 bands when the cells were co-treated with 20 μU/ml LH (Figure 2B).

Dose response of IGF-I and LH

When TIC were cultured for 2 days with varying concentrations of IGF-I (0.1 to 100 ng/ml), there was a dose-dependent increase ($ED_{50} = 12.4 \pm 3.3$ ng/ml) in the accumulation of

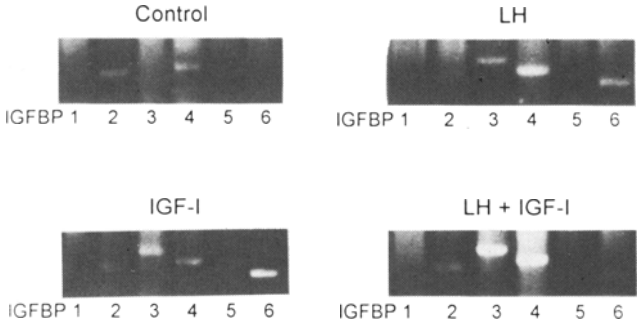

Figure 1 Expression of IGFBP-1, -2, -3, -4, -5 and -6 mRNAs in ovarian theca-interstitial cells. Isolated theca-interstitial cells (3×10^5 viable cells/well) were cultured (2d) in 96-well tissue culture plates containing 0.2 ml of medium with and without IGF-I (30 ng/ml) and LH (20 μIU/ml). IGFBP mRNAs were amplified from cytoplasmic RNA extracts of the cultured cells by specific RT-PCR. The amplification products were separated on a 2% agarose gel and stained with ethidium bromide

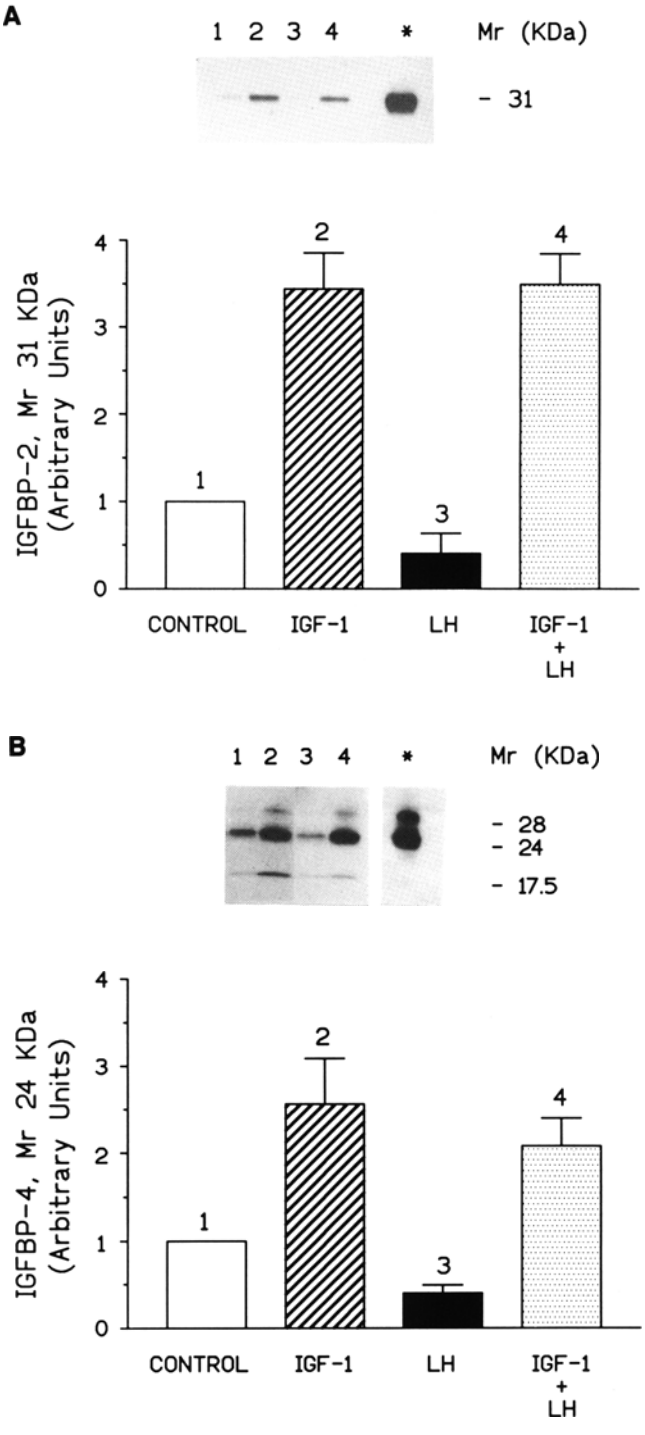

Figure 2 Western immunoblotting of IGFBP-2 and -4 in conditioned media. Rat theca interstitial cells were cultured for 2 days in serum-free medium as control (no additions) or with 20 μU/ml LH and/or 30 ng/ml IGF-I. After culture, the IGFBPs were analysed by Western immunoblotting using specific antisera for IGFBP-2 (**A** panel) and IGFBP-4 (**B** panel). Data are mean ± SEM ($n = 3$). Typical Western blot results are shown; *, 20 ng of rat IGFBP-2 and IGFBP-4 were loaded onto the respective gels

IGFBP-2 (Figure 3A) and IGFBP-4 (Figure 3B) in the conditioned media. In the case of IGFBP-4 (Figure 3B), the 17.5 kDa fragment was detectable only at maximal concentrations of IGF-I ($\geq$ 30 ng/ml). Treatment with graded doses of LH (0.2-200 μU/ml) caused small variable changes in basal IGFBP-2 and -4 levels, but the changes were not statistically significant at any dose (data not shown).

Effect of LH on the IGF-I dose response curve

As seen in Figure 5, co-treatment with a high dose of LH (20 μU/ml) did not significantly change ($P > 0.05$) the dose response relationship between IGF-I ($ED_{50} = 13.4 \pm 4.4$ ng/ml) and the production of either the 31 kDa IGFBP-2 (Figure 4A), or the 24 kDa and 17.5 kDa IGFBP-4 forms (Figure 5B). In some experiments (see Figure 4B, Western immunoblot for example), co-treatment with LH appeared to enhance the potency of IGF-I; however, the effect was not statistically significant ($P > 0.05$).

Effect of IGF-I on the LH dose response curve

As seen in Figure 5, the stimulatory effect of a saturating dose of IGF-I (30 ng/ml) on IGFBP-2 (Figure 5A) and IGFBP-4 (Figure 5B) was not significantly affected by co-incubation with graded doses of LH. However, in some experiments, the production of the 28 kDa, 24 kDa and 17.5 kDa IGFBP-4 bands by IGF-I appeared to be enhanced by LH concentrations ≥ 2 μU/ml (Figure 5B). But, these effects were not statistically significant ($P > 0.05$).

Discussion

This serum-free tissue culture system is a physiologically relevent model for studying TIC cytodifferentiation (Erickson *et al.*, 1985). We have demonstrated that IGFBP-2, -3, -4 and -6 mRNAs are expressed in cultured TIC. The 31 kDa IGFBP-2 protein, the 24 and 28 kDa forms of IGFBP-4 and

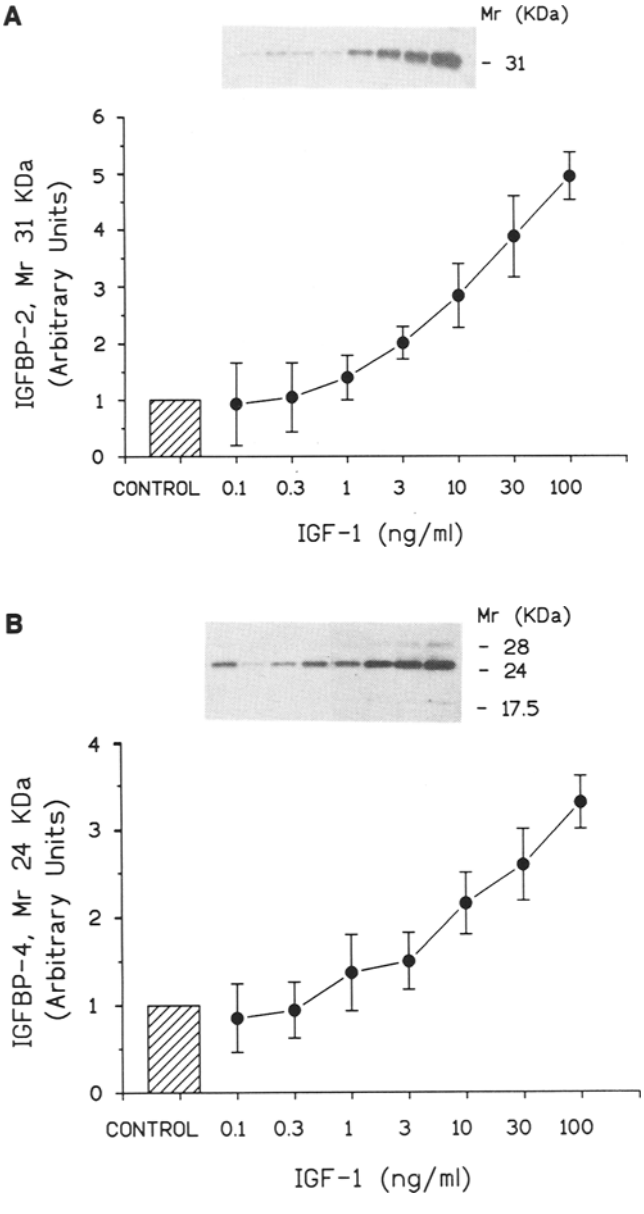

Figure 3 Dose dependency of IGFBP production by theca interstitial cells in response to IGF-I. Cells were cultured for 2 days in serum free medium with the indicated concentrations of IGF-I. The levels of IGFBP-2 (**A** panel) and IGFBP-4 (**B** panel) in conditioned media were measured after Western immunoblotting. Data are mean $\pm$ SEM ($n = 3$). The Western blot results are shown

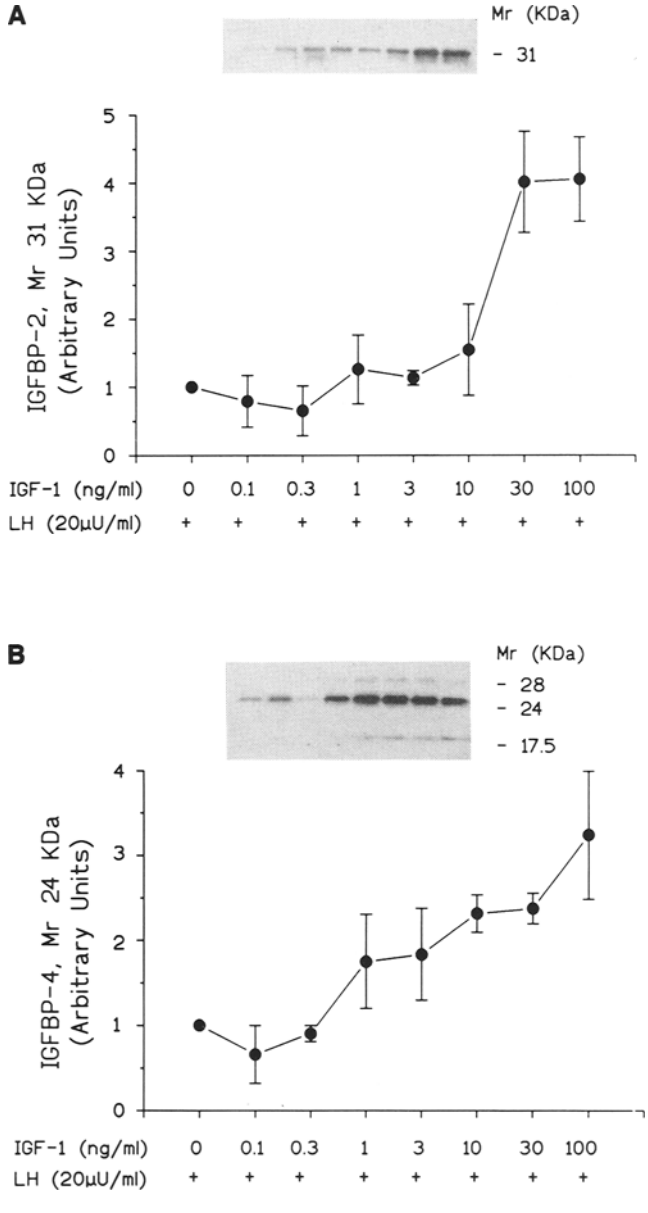

Figure 4 Effect of LH on the IGF-I dose response curve. Cells were cultured for 2 days in serum-free medium with the indicated concentrations of IGF-I and LH, after which the conditioned media were analysed for the IGFBP-2 (**A** panel) and IGFBP-4 (**B** panel) by Western immunoblotting. Data are mean $\pm$ SEM ($n = 3$). The Western immunoblot results are shown

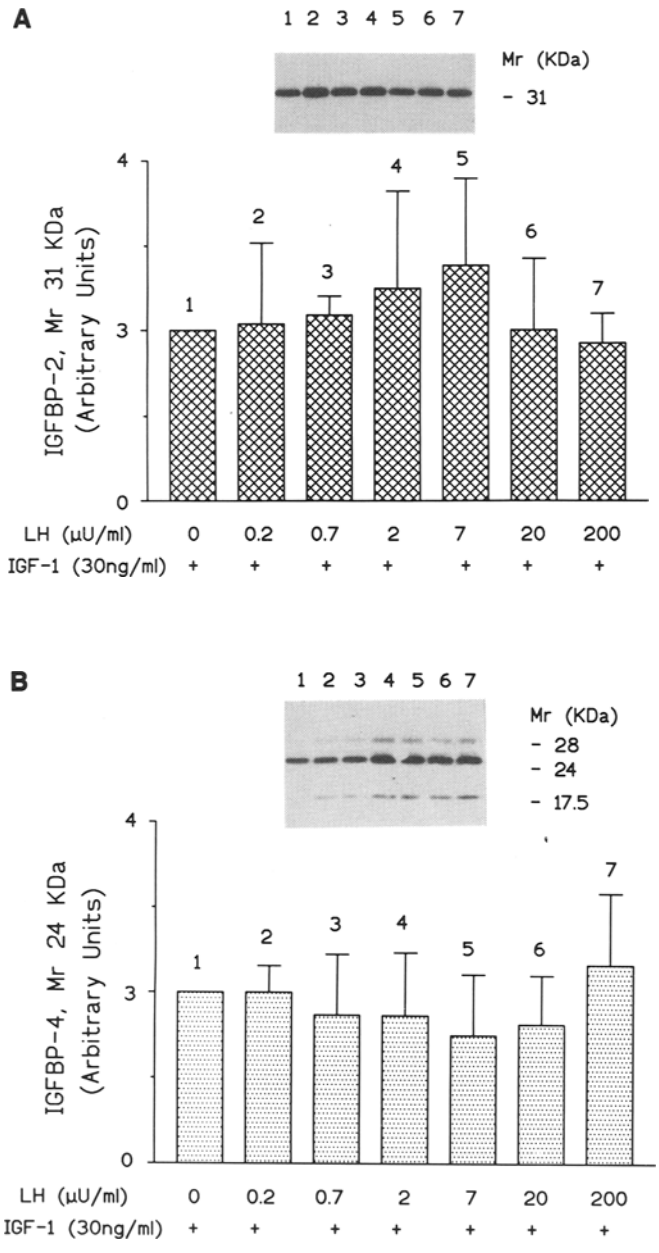

Figure 5 Effect of IGF-I on the LH dose response curve. Cells were cultured for 2 days in serum-free medium with the indicated concentrations of LH and IGF-1. The conditioned media were removed and assayed for IGFBP-2 (**A** panel) and IGFBP-4 (**B** panel) by Western immunoblotting. The Western immunoblot results are shown

a putative IGFBP-4 protease were secreted by TIC, but there was very little or no IGFBP-3, -5 or -6 proteins in the conditioned medium. A stimulatory role for IGF-I in the expression of IGFBP-2, and -4 was established. By contrast, LH did not significantly effect the expression of TIC IGFBPs. Together, these data suggest that IGF-I, possibly derived from granulosa cells, may function as a dominant stimulator of the rat TIC IGFBP system. Because IGF-I is a potent stimulator of TIC function, this stimulation could influence the level of ovary androgen production which in turn could influence folliculogenesis and atresia.

Transcripts for IGFBP-2, -3, -4 and -6 were detected by RT-PCR in cultured TIC. These data confirm and extend previous reports using solution hybridization techniques (Ricciarelli *et al.*, 1991, 1992; Rohan *et al.*, 1993). The identification of IGFBP-2 mRNA is physiologically significant because TIC express high levels of IGFBP-2 transcripts during normal folliculogenesis (Erickson *et al.*, 1994b). Similarly, the expression of IGFBP-4 mRNA in rat TIC *in situ* (Erickson *et al.*, 1992a) argues that the identification of IGFBP-4 transcripts in cultured TIC may be physiologically relevant. The interpretation of IGFBP-3 and IGFBP-6 transcripts is more complex because these mRNAs have not been identified in rat TIC *in situ* (Erickson *et al.*, 1994a). This apparent descrepancy could result from the differences in sensitivity between the *in situ* hybridization and RT-PCR techniques. Alternatively there could be other explanations. In the case of IGFBP-3, the mRNA is restricted to corpora lutea, being present in theca and granulosa lutein cells and blood tissue (Erickson *et al.*, 1993). In addition, IGFBP-3 mRNA is not detectable in TIC of immature intact or hypophysectomized rats *in situ* (Erickson, unpublished result). The evidence that IGFBP-3 transcripts were found in LH, but not IGF-I treated cultures, suggests a specific role for LH in the expression of IGFBP-3, perhaps associated with the luteinization pathway. In the case of IGFBP-6, the mRNA (Erickson *et al.*, 1994a) and protein (Erickson, unpublished result) are selectively expressed in smooth muscle and stroma cells in the rat ovary. The purified TIC preparation is known to contain ~5% non TIC (Magoffin & Erickson, 1988a). Thus, contaminating smooth muscle and stroma might also be responsible for the IGFBP-6 mRNA. Finally, the concept of non coordinate regulation of the synthesis of IGFBP-3 and -6 mRNAs and proteins is suggested by our data. This evidence could indicate some translational control mechanisms for IGFBP-3 and -6.

We have demonstrated the 31 kDa IGFBP-2 protein in TIC conditioned medium. Ricciarelli *et al.* (1991) reported a similar finding. These results demonstrate the important concept that rat TIC indeed synthesize and secrete mature IGFBP-2 protein. Immunoreactive IGFBP-2 is also found in TIC in developing follicles (Erickson, unpublished result). Thus, the 31 kDa IGFBP-2 produced by TIC may be a physiologically relevent protein. It is interesting that control (untreated) TIC secrete relatively high levels of 31 kDa IGFBP-2 during the 48 h culture period. The manner in which this spontaneous production of IGFBP-2 is accomplished remains unknown. It may be that rat TIC produce IGFBP-2 constitutively. Consistent with this idea is the presence of relatively high levels of IGFBP-2 mRNA in TIC after hypophysectomy (Erickson, unpublished result). Although not conclusive the concept is emerging that IGFBP-2 might be a major molecular marker for rat TIC cytodifferentiation.

The function of IGFBP-2 is not known; however, there is evidence that IGFBP-2 may function as an antigonadotropin. A dramatic inhibitory effect of IGFBP-2 on FSH induced estradiol and progesterone production by rat granulosa cells has been reported (Bicsak *et al.*, 1990). This observation supports the hypothesis that the IGFBP-2 secreted by TIC might decrease the ability of FSH to function physiologically. One mechanism by which IGFBP-2 blocks FSH is by binding intrinsic IGF-I thereby inhibiting IGF-I from interacting with its receptor; another mechanism appears to involve the binding of IGFBP-2 to the cell surface (Bicsak *et al.*, 1990). Rat IGFBP-2 which contains 270 amino acids (predicted Mr 29 564) and has no potential N-linked glycosylation sites, contains the Arg-Gly-Asp (RGD) tripeptide motif near the C-terminus (Brown *et al.*, 1989; Margot *et al.*, 1989). The RGD sequence is of interest because many proteins with this motif (including hormones and growth factors) can bind to integrin receptors (Hynes, 1987), and the binding event can stimulate signaling pathways including protein tyrosine kinase (Akiyama *et al.*, 1994). This evidence raises the intriguing possibility that TIC derived IGFBP-2 may bind to integrin receptors on granulosa and/or TIC, which in turn might elicit biological responses. Experiments are in progress to test this possibility.

We present the first evidence that rat TIC secrete IGFBP-4. Both the 24 and 28 kDa forms of IGFBP-4 were found in the conditioned media, but the amount of the 24 kDa form

was much greater. Rat IGFBP-4 contains 233 amino acids with a predicted molecular weight of 25.7 kDa and has one potential N-linked glycosylation site (Shimasaki *et al.*, 1990). The 24 and 28 kDa bands correspond to the nonglycosylated and glycosylated form of IGFBP-4 respectively (Shimonaka *et al.*, 1989; Mohan *et al.*, 1989; Ceda *et al.*, 1991). The observation that 24 kDa IGFBP-4 is the predominant form secreted by cultured TIC is consistent with other studies of cultured rat cells, including rat granulosa cells (Liu *et al.*, 1993) and a rat neuroblastoma cell line (Ceda *et al.*, 1991). The non glycosylated 24 kDa IGFBP-4 may therefore be the physiologically relevant protein in the rat. Although rat granulosa cells are capable of secreting 24 and 28 kDa IGFBP-4 *in vitro* (Liu *et al.*, 1993), it is unlikely that they make a significant contribution to the IGFBP-4 in this study because (1) the purified TIC preparations do not contain granulosa cells (Magoffin & Erickson, 1988b) and (2) high doses of IGF-I decrease IGFBP-4 expression in rat granulosa cells (Liu & Ling, 1994), which is the exact opposite of our present results. The physiological significance of IGFBP-4 secretion by TIC is unknown; however several studies have implicated IGFBP-4 in follicle atresia (Liu *et al.*, 1993; Erickson *et al.*, 1994a). In this respect, immunocytochemical analysis of IGFBP-4 *in vivo* indicates that TIC in atretic follicles strongly express IGFBP-4 protein (Erickson, unpublished result). This evidence, together with the well documented concept that TIC are able to activate atresia *in vivo* (Erickson *et al.*, 1985), leads us to propose the novel hypothesis that the 24 kDa IGFBP-4 secreted by TIC may be an atretogenic signal.

The conditioned media contained a 17.5 kDa immunoreactive IGFBP-4 fragment. This implies that rat TIC express a proteolytic enzyme that hydrolyzes the mature IGFBP protein. Although the nature of this putative protease is unknown, experiments with other cell types have identified IGFBP-4 protease. It is a cation-dependent serine protease which hydrolyzes mature IGFBP-4 proteins into 17.5 kDa and 14 kDa fragments (Cheung *et al.*, 1994). The protease is specific for IGFBP-4 and does not cleave the other forms of IGFBPs (Conover *et al.*, 1993; Kanzaki *et al.*, 1994). The absence of immunoreative IGFBP-2 fragments in the TIC cultures is consistent with the enzyme specificity. The catalytic activity of the IGFBP-4 protease appears to be tightly regulated. It appears that IGFBP-4 protease is secreted in an inactive form and activation occurs in association with IGF binding (Conover *et al.*, 1993). Also, a yet unidentified IGFBP-4 protease inhibitor has the capacity to rapidly and effectively block IGF-I dependent IGFBP-4 protease (Conover *et al.*, 1993). Further work is needed to establish the possible role of IGF-I and the putative inhibitor in regulating TIC IGFBP-4 protease activity. An important question concerns the function of this putative IGFBP-4 protease. It is clear that hydrolysis of IGFBP-4 by the protease decreases IGF-I affinity dramatically (Liu *et al.*, 1993; Cheung *et al.*, 1994). Accordingly TIC IGFBP-4 protease may be implicated in regulating the level of free IGF-I in the follicle wall. If true, it could have interesting implications for the control of atresia.

A stimulatory role for IGF-I in the expression of IGFBP-2, and IGFBP-4 in TIC was established. This is the first evidence which supports the concept that the TIC IGFBP system is regulated by growth factors. The ability of nM concentrations of IGF-I to evoke these stimulations suggests they may be physiologically significant. The mechanism whereby IGF-I stimulates the TIC IGFBP system is unknown. Presumably, it relates to the activation of the IGF-I receptor protein tyrosine kinase signal transduction pathway in the TIC (Magoffin & Erickson, 1994). The capacity for IGF-I to stimulate IGFBP production in other cell types has been reported i.e, IGFBP-3 (Grimes & Hammond, 1992) and IGFBP-3 protease (Grimes & Hammond, 1994) in porcine granulosa cells, IGFBP-4 and IGFBP-4 protease in human fibroblasts (Camacho-Hubner, 1992) and rat nerve cells (Cheung *et al.*, 1994), and IGFBP-5 in rat granulosa cells (Adashi *et al.*, 1994). This stimulatory effect is not universal, however since IGF-I inhibits the expression of IGFBP-2 in rat hepatocytes (Boni-Schnetzler *et al.*, 1990), and IGFBP-4 (Liu *et al.*, 1993), and IGFBP-5 protease (Fiedler *et al.*, 1993) in rat granulosa cells. Therefore, the way in which IGF-I modulates the IGFBP system appears to be cell specific.

Given that the IGFBPs change in a similar manner, it seems likely that IGF-I regulates the expression of this group of proteins in TIC coordinately. The ability of IGF-I to regulate the expression of P450scc, 3β SDH and LH receptor in a concerted fashion has also been reported (Magoffin & Erickson, 1994). Together, these results suggest that a major role of IGF-I in TIC is to selectively increase the expression of key groups of genes, including specific IGFBPs. Presumably, this is important in specifying the proper sequence and timing of TIC differentiation. The physiological importance of IGF-I in folliculogenesis is emphasized by the fact that rat granulosa cells in healthy follicles strongly express IGF-I (Hernandez *et al.*, 1989, 1990; Oliver *et al.*, 1989; Zhou *et al.*, 1991). Thus, a possible role for granulosa IGF-I in regulating the TIC IGFBP system is suggested.

Despite the main role of LH in stimulating TIC cytodifferentiation (Erickson *et al.*, 1985), LH appeared to be inactive in regulating the synthesis of the TIC IGFBP proteins. The inability of LH to significantly alter IGFBP protein levels was unrelated to LH concentration or the presence of IGF-I in the medium. One is led to the conclusion, therefore, that LH may not be a physiologically important hormone in controlling IGFBP protein synthesis in rat TIC. In contrast, LH did increase the relative amounts of IGFBP-3 and -6 mRNA in TIC. This suggests that complex relationships may exist between LH and the transcription and translation of the IGFBP mRNAs. It has been shown that FSH causes biphasic effects on IGFBP-4 (Liu *et al.*, 1993; Erickson *et al.*, 1994) and IGFBP-5 (Adashi *et al.*, 1991; Onoda *et al.*, 1995) mRNA and protein synthesis. These FSH effects appear to be mediated by the cyclic AMP/protein kinase A signal transduction pathway (Adashi *et al.*, 1993). Thus, it seems that different types of signaling pathways regulate IGFBP-4 expression in the rat TIC and granulosa cells. The physiological significance for the different regulation remains to be determined.

What might these IGF-I stimulations imply for TIC function? We have demonstrated that IGF acts synergistically with LH to control the level of androgen production by TIC (Magoffin & Erickson, 1988a). The IGF-I stimulation of IGFBP production by TIC could function in modulating the bioactivity of the IGF-I which inturn could modulate the level of androgen production. This modulatory effect could have implications for folliculogenesis and atresia, as well as ovarian disorders such as hyperandrogenism.

Materials and methods

Reagents

Ovine LH (AFP-555 1B; 2.3 NIH-LH-S1 U/mg) was a gift from The National Hormone and Pituitary Program of the NIDDK, Rockville, MD and recombinant human IGF-I was obtained from AMGEN (Thousand Oaks, CA). m-MLV reverse transcriptase, vanadyl ribonucleoside complexes and proteinase K were obtained from Gibco BRL (Gaithersburg, MD, USA). RNAsin was obtained from Promega (Madison, WI, USA). Reagents for polymerase chain reactions including Taq DNA polymerase were obtained from Perkin-Elmer Cetus (Norwalk, CT, USA).

Animals

Immature female Sprague-Dawley rats were hypophysectomized by Harlan Sprague-Dawley (Harlan Industries,

Indianapolis, IN) at 21 days of age. Animals were given 5% dextrose and food *ad libitum*. Five days after hypophysectomy the rats were killed by cervical dislocation after having been rendered unconscious by CO_2 inhalation as approved by the Institutional Animal Care and Use Committee. The ovaries were collected in ice-cold medium 199 containing 1 mg/ml. BSA and 25 mM HEPES.

Cell culture

The ovaries were dispersed with collagenase and deoxyribonuclease as previously described (Magoffin & Erickson, 1982). The theca-interstitial cells (TIC) were isolated by discontinuous Percoll gradient centrifugation (Magoffin & Erickson, 1988b). TIC ($2-5 \times 10^4$ viable cells/well), free of granulosa contamination, were cultured in 96-well tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ)) in 0.2 ml McCoy's 5a medium (Gibco supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 2 mM L-glutamine with and without LH (0–20 μU/ml) and IGF-I (0–100 ng/ml). The TIC were cultured for 2 days in a humidified 95% air, 5% CO_2 atmosphere at 37°C. The conditioned medium was collected and frozen (−20°C) until assayed for androsterone by RIA and IGFBPs by Western immunoblotting.

Western blotting analysis

Samples of conditioned media were concentrated and analysed by Western blotting using specific polyclonal antibodies to rat IGFBP-1, -2, -3, -4, -5 and -6 as described (Liu *et al.*, 1993; Erickson *et al.*, 1994c). SDS–PAGE was performed by using the buffer system of Laemmli in the absence of sulfhydryl reducing agents using the Xcell minicell system (Novex, Sorrento Valley, CA). Conditioned media was concentrated 10 times by using Ultrafree-MC filters (10 000 NMWL Millipore, MA). Samples were loaded onto 12% polyacrylamide gel (Tris/glycine percentage gel, Novex, CA) electrophoresed at constant voltage 100 V for 2 h and then transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Richmond, CA) at constant current 150 mA for 2 h using the Mini Blot module (Novex, CA).

Immunoblotting was performed by first treating the nitrocellulose membrane (Bio-Rad) with 1% casein/TBST (Tris buffered saline tween- 20) buffer for 1 h at room temperature. Then the membrane was incubated with diluted IGFBP antisera in 1% casein/TBST buffer overnight at 4°C. The IGFBP antisera were used at 1:1000 dilution. The membrane was washed three times with TBST buffer and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Calbiochem) diluted 1:10 000 in 1% casein/TBST for 2 h at room temperature. The membrane was washed five times with TBST buffer, after which it was incubated with luminol and H_2O_2 for 1 min in the dark by using the ECL-Western blotting detection reagents (Amersham, Arlington Heights, IL). After incubation, the membrane was exposed to Hyperfilm-ECL (Amersham) for 1–10 min. After chemiluminescence, the films were scanned by laser densitometry.

Measurement of mRNA

mRNA was measured (Sambrook *et al.*, 1989) by transcribing the mRNA into cDNA using reverse transcriptase (RT) followed by amplification of the cDNA using the polymerase chain reaction (PCR). After the medium was collected from the TIC cultures the cells were lysed in 50 μl ice cold RNA extraction buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH = 8.6, 0.5% Nonidet P-40, 1 mM dithiothreitol, 20 mM vanadyl ribonucleoside complexes). The extracts from four replicate wells were pooled and centrifuged (14 500 *g*) for 90 s. The supernatant was diluted with 200 μl proteinase K digestion buffer (0.2 M Tris-HCl, pH = 8.0, 25 mM EDTA, 0.3 M NaCl, 2% sodium dodecyl sulfate) and 20 μg proteinase K was added. The mixture was digested (37°C) for 30 min then extracted with 400 μl phenol/chloroform. The RNA was precipitated with 400 μl isopropanol and the pellet was resuspended in 20 μl water then frozen (−80°C). Aliquots of RNA (4 μl) were transcribed into cDNA by incubating in 20 μl of 10 mM Tris-HCl, pH = 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 5 μg oligo$(dT)_{12-18}$, 20 units RNAsin, 200 units M-MLV reverse transcriptase for 30 min at 37°C. The reaction was then heated to 95°C (5 min) and cooled at 4°C. 50 pmol of each PCR primer, 8 μl 10X PCR buffer (100 mM Tris-HCl, pH = 8.3, 500 mM KCl), 6 μl 25 mM $MgCl_2$ and 2.5 units Taq DNA polymerase were added and the volume adjusted to 80 μl. The cDNA was amplified for 30 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) in a thermal cycler. The amplification products were ethanol precipitated then separated on a 2% agarose gel. The DNA was visualized with ethidium bromide staining. The primers were specific for each binding protein as determined by specificity studies using full length rat IGFBP cDNA clones as templates. The primers for IGFBP-1 (5′-GAG CCT CGA CCT CTG CAT GC-3′; 5′-GCA GAG CCC AGC TTC TCC AT-3′) amplified a 450 bp fragment (Murphy *et al.*, 1990), the IGFBP-2 primers (5′-CGA GCA GTA TCC CCT GAA GG-3′; 5′-CTT ATA GAA CCC CTT CTT GT-3′) amplified a 442 bp product (Brown & Rechler, 1990), the IGFBP-3 primers (5′-AAC ACC ACT GAG TCT GAG GA-3′; 5′-CAC AGC GGT ATC TAC TGG CT-3′) amplified a 482 bp product (Shimasaki *et al.*, 1989), the IGFBP-4 primers (5′-CGC TGT GGC TCA GGC ATG GC-3′; 5′-TCC AGA GCA GGA TGA CAC TG-3′) amplified a 431 bp fragment (Shimasaki *et al.*, 1990), the IGFBP-5 primers (5′-ATG GCT GAG GAG ACC TAC TC-3′; 5′-TGC GCA GTG AGT TGC AAT GA-3′) amplified a 446 bp fragment (Shimasaki *et al.*, 1991b), and the IGFBP-6 primers (5′-GTC TAC ATC CCT AAG TGC GC-3′; 5′-AAC GAC ACT GCT GCT TGC GG-3′) amplified a 373 bp product (Shimasaki *et al.*, 1991a).

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References

Adashi, E.Y., Resnick, C.E., Hurwitz, A., Ricciarelli, E., Hernandez, E.R. & Rosenfeld, R.G. (1991). *Endocrinology*, **128,** 754–760.

Adashi, E.Y., Resnick, C.E., Tedeschi, C. & Rosenfeld, R.G. (1993). *Endocrinology*, **132,** 1463–1468.

Adashi, E.Y., Resnick, C. & Rosenfeld, R.G. (1994). *Molec. Cell Endocrinol.*, **99,** 279–284.

Akiyama, S.K., Yamada, S.S., Yamada, K.M., La Flamme, S.E. (1994). *J. Biol. Chem.*, **269,** 15961–15964.

Bicsak, T., Shimonaka, M., Malkowski, M. & Ling, N. (1990). *Endocrinology*, **126,** 2184–2189.

Boni-Schnetzler, M., Schmid, C., Mary, J.-L., Zimmerli, B., Meier, P., Zapf, J., Schwander, J. & Foresch, E. (1990). *Molec. Endocrinol.*, **4,** 1320–1326.

Brown, A., Chiariotti, L., Orlowski, C., Mehlman, T., Burgess, W., Ackerman, E., Bruni, C. & Rechler, M. (1989). *J. Biol. Chem.*, **264,** 5148–5154.

Brown, A. & Rechler, M. (1990). *Molec. Endocrinol.*, **4,** 2039–2051.

Camacho-Hubner, C., Busby, Jr. W.H., McCusker, R.H., Wright, G. & Clemmons, D.R. (1992). *J. Biol. Chem.*, **267,** 11949–11956.

Cara, J. & Rosenfeld, R. (1988). *Endocrinology*, **123,** 733–739.

Ceda, G.P., Fielder, P.J., Henzel, W.J., Louie, A., Donovan, S.M., Hoffman, A.R. & Rosenfeld, R.G. (1991). *Endocrinology*, **128,** 2815–2824.

Cheung, P., Wu, J., Banach, W. & Chernausek, S. (1994). *Endocrinology*, **135,** 1328–1335.

Conover, C., Clarkson, J. & Bale, L. (1993). *Endocrinology*, **133,** 1347–1351.
Erickson, G., Magoffin, D., Dyer, C. & Hofeditz, C. (1985). *Endocrine Reviews*, **6,** 371–399.
Erickson, G., Nakatani, A., Ling, N. & Shimasaki, S. (1992). *Endocrinology*, **130,** 625–636.
Erickson, G. (1993). *Seminars in Reprod. Endocrinol.*, **11,** 307–312.
Erickson, G., Nakatani, A., Ling, N. & Shimasaki, S. (1993). *Endocrinology*, **133,** 1147–1157.
Erickson, G., Nakatani, A., Liu, X.-J., Shimasaki, S. & Ling, N. (1994a). The role of IGF-I and the IGFBPs in folliculogenesis. *Molecular Biology of the Female Reproductive System.* Findlay, J.K. (ed). Academic Press, pp. 101–127.
Erickson, G., Mitchell, C., Nakatani, A., Ling, N. & Shimasaki, S. (1994b). *Endocrine Journal*, **2,** 447–456.
Erickson, G., Li, D., Sadrkhanloo, R., Liu, X.-J., Shimasaki, S. & Ling, N. (1994c). *Endocrinology*, **134,** 1365–1372.
Fiedler, P.J., Pham, H., Adashi, E.Y. & Rosenfeld, R.G. (1993). *Endocrinology*, **133(1),** 415–418.
Grimes, R. & Hammond, J. (1992). *Endocrinology*, **131,** 553–558.
Grimes, R. & Hammond, J. (1994). *Endocrinology*, **134,** 337–343.
Hernandez, E.R., Resnick, C.E., Svoboda, M.E., Van Wyk, J.J., Payne, D.W. & Adashi, E.Y. (1988). *Endocrinology*, **122,** 1603–1612.
Hernandez, E., Roberts, C., LeRoith, D. & Adashi, E. (1989). *Endocrinology*, **125,** 572–574.
Hynes, R.O. (1987). *Cell*, **48,** 549–554.
Kanzaki, S., Hilliker, S., Baylink, D. & Mohan, S. (1994). *Endocrinology*, **134,** 383–392.
Ling, N., Ui, M., Shimonaka, M., Shimasaki, S. & Bicsak, T. (1990). An IGF-binding protein in ovarian follicular fluid blocks FSH-stimulated steroid production by ovarian granulosa cells. *Neuroendocrine Regulation of Reproduction.* Yen, S.S.C., Vale, W. (eds). Serono Symposia Publ: New York. pp. 207–215.
Liu, X.-J., Malkowski, M., Guo, Y.-L., Erickson, G., Shimasaki, S. & Ling, N. (1993). *Endocrinology*, **132,** 1176–1183.
Liu, X.-J. & Ling, N. (1994). *Advances Experimental Medicine Biology*, **343,** 367–376.
Magoffin, D. & Erickson, G. (1982). *J. Bio. Chem.*, **257,** 4507–4513.
Magoffin, D. & Erickson, G. (1988a). *In Vitro Cell Devel. Biol.*, **24,** 862–870.
Magoffin, D. & Erickson, G. (1988b). *Endocrinology*, **122,** 2345–2347.
Magoffin, D., Kurtz, K. & Erickson, G. (1990). *Molec. Endocrin.*, **4,** 489–496.
Magoffin, D. & Weitsman, S. (1993a). *Molec. Cell Endocrin.*, **96,** 45–51.
Magoffin, D. & Weitsman, S. (1993b). *Biol. Reprod.*, **48,** 1166–1173.
Magoffin, D. & Weitsman, S. (1993c). *Endocrinology*, **132,** 1945–1951.
Magoffin, D. & Erickson, G. (1994). Control systems of theca and interstitial cells. (J.K. Findlay, ed.) *Molecular Biology of the Female Reproductive System.* Academic Press, pp. 39–65.
Magoffin, D. & Weitsman, S. (1994). *Biol. Reproduc.*, **51,** 766–775.
Margot, J., Binkert, C., Mary, J.-L., Landwehr, J., Heinrich, G. & Schwander, J. (1989). *Mol. Endocrinol.*, **3,** 1053–1060.
Mohan, S., Bautista, C., Wergedal, J. & Baylink, D. (1989). *Proc. Natl. Acad. Sci.*, **86,** 8338–8342.
Murphy, L., Seneviratine, C., Ballejot, G., Croze, F. & Kennedy, T. (1990). *Mol. Endocrinol.*, **4,** 329–336.
Nakatani, A., Shimasaki, S., Erickson, G. & Ling, N. (1991). *Endocrinology*, **129,** 1521–1529.
Oliver, J., Aitman, T., Powell, J., Wilson, C. & Clayton, R. (1989). *Endocrinology*, **124,** 2671–2679.
Onoda, N., Li, D., Mickey, G., Erickson, G. & Shimasaki, S. (1994). *Molec. Endocrinol.*, (in press).
Rechler, M. (1993). *Vitam. Horm.*, **47,** 1–114.
Ricciarelli, E., Hernandea, E., Hurwitz, A., Kokia, E., Rosenfeld, R., Schwander, J. & Adashi, E. (1991). *Endocrinology*, **129,** 2266–2268.
Ricciarelli, E., Hernandez, E., Tedeschi, C., Botero, L., Kokia, E., Rohan, R., Rosenfeld, R., Albiston, A., Herington, A. & Adashi, E. (1992). *Endocrinology*, **130,** 3092–3094.
Rohan, R., Ricciarelli, E., Kiefer, M., Resnick, C. & Adashi, E. (1993). *Endocrinology*, **132,** 2507–2512.
Sambrook, J., Fritsch, E., Maniatis, T. (1989). Molecular cloning: a laboratory manual. Sambrook, J., Fritsch, E. & Maniatis, T. (eds.), 2nd edition. Cold Spring Harbor Press, pp. 14–18.
Shimasaki, S., Koba, A., Mercado, M., Shimonaka, M. & Ling, N. (1989). *Biochem. Biophys. Res. Commun.*, **65,** 907–912.
Shimasaki, S., Uchiyama, F., Shimonaka, M. & Ling, N. (1990). *Mol. Endocrinol.*, **4,** 1451–1458.
Shimasaki, S., Gao, L., Shimonaka, M. & Ling, N. (1991a). *Molec. Endocrinol.*, **5,** 938–948.
Shimasaki, S., Shimonaka, M., Zhang, H.-P. & Ling, N. (1991b). *J. Biol. Chem.*, **266,** 10646–10653.
Shimasaki, S. & Ling, N. (1992). *Prog. in Growth Factor Res.*, **3,** 243–266.
Shimonaka, M., Schroeder, R., Shimasaki, S. & Ling, N. (1989). *Biochem. Biophys. Res. Commun.*, **165,** 189–195.
Zhou, J., Chin, E. & Bondy, C. (1991). *Endocrinology*, **129,** 3281–3288.