0163-769X/99/$03.00/0
Endocrine Reviews 20(4): 535–582
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The Insulin-Related Ovarian Regulatory System in Health and Disease

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I. Introduction

INSULIN, a pancreatic peptide hormone produced in the β-cells of the islets of Langerhans, plays a major role in the regulation of carbohydrate, fat, and protein metabolism (1). The classical target organs for insulin action are muscle, adipose tissue, and liver (2). Until approximately a decade ago, insulin was not thought to play a significant role in the regulation of ovarian function, despite suggestions of the "gonadotropic" function of insulin (3) in observations of abnormal ovarian function in young women with type 1 diabetes mellitus by Joslin *et al*. (4), which predated the discovery of insulin more than 75 years ago (5). A resurgence of interest in the ovarian effects of insulin was stimulated by observations of severe ovarian hyperandrogenism in women with syndromes of extreme insulin resistance (6, 7), which led to the hypothesis that high levels of circulating insulin may cause excessive androgen production in these patients (8, 9). The demonstration of insulin's ability to stimulate steroidogenesis in ovarian cells *in vitro* (10) and the demonstration of insulin receptors in both stromal and follicular compartments of the human ovary (11, 12) established the ovary as another important target organ for insulin action.

This field was further expanded by studies of the ovarian production and ovarian effects of the insulin-like growth factors, IGF-I and IGF-II, by the discovery of ovarian type I and type II IGF receptors, and by the discovery of the ovarian production of binding proteins [IGF-binding proteins (IGFBPs)] for these two growth factors (13–15). Thus, in addition to insulin, a role for the structurally related IGFs in ovarian function has gained recognition. Over the last decade, a significant amount of information has accumulated about the role of insulin and IGFs in the ovary at the molecular, cellular, and clinical levels in a variety of normal and pathological conditions. Therefore, a need has arisen for a comprehensive review of what we term the insulin-related ovarian regulatory system. This system consists of the following components (Table 1): insulin; IGF-I and IGF-II; insulin receptor; type I and type II IGF receptors; IGFBPs 1–6; and IGFBP proteases.

While the pituitary ovarian regulators, LH and FSH, are of paramount importance to ovarian function (16, 17), the insulin-related ovarian regulatory system likewise participates in normal follicle development (3, 14, 18–23). Its alterations may be important in the ovarian dysfunctions observed in a number of disorders, including diabetes mellitus, obesity, polycystic ovary syndrome (PCOS), and syndromes of extreme insulin resistance (9, 24–28). The physiological and

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* This work was supported in part by NIH Grants M01 RR-00047 (L.P.), NICHD R035618–01A1 (L.P. and Z.R.), K08 HD-01141 (N.A.C.) and R01 HD-31579 (L.C.G.).

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TABLE 1. Components of the insulin-related ovarian regulatory system

Insulin
IGF-I
IGF-II
Insulin receptor
Type I IGF receptor
Type II IGF receptor
IGFBPs 1–5
IGFBP proteases

clinical significance of this regulatory system is underscored by recent observations which demonstrate that pharmacological agents capable of manipulating the components of this system may be useful in the therapy of some of these disorders (29–38).

This article reviews the role of each component of the insulin-related ovarian regulatory system in both normal ovarian physiology and in relevant pathological states, the interactions among the components of this system, and the therapeutic implications of this system for women with abnormal ovarian function.

II. Insulin and Insulin Receptor

A. Structures of insulin and insulin receptor

Detailed reviews of the structures of insulin and its receptor are available (1, 2, 39–42), and thus only a brief overview will be presented here.

Insulin is a 5900 mol wt polypeptide secreted by the β-cells of the pancreatic islets of Langerhans. The human insulin gene is located on chromosome 11 (39) and encodes preproinsulin, a 110-amino acid single-chain polypeptide that is the precursor of insulin (1). Pre-proinsulin is proteolytically converted to proinsulin, which consists of the A chain, B chain, and C peptide. Proinsulin is homologous with IGF-I and -II and can bind to the insulin receptor with approximately 10% of the affinity of insulin. Insulin is produced after the C-peptide is cleaved from proinsulin by endopeptidases active in the Golgi apparatus and in secretory granules. The endopeptidases preferentially cleave either at the C peptide/B chain junction, between Arg31 and Arg32 (endopeptidase type I), or at the C peptide/A chain junction, between Lys64 and Arg65 (endopeptidase type II). The resulting insulin molecule consists of an A chain (21 amino acids) and a B chain (30 amino acids), with three disulfide bridges: two between the A and the B chains (A7-B7 and A20-B12) and one within the A chain (A6-A11).

The insulin receptor is a heterotetramer consisting of two α- (135 kDa molecular mass) and two β- (95 kDa molecular mass) subunits (2). The gene for the insulin receptor is located on the short arm of chromosome 19 (43–45), contains 22 exons, is more than 150 kb in length, and encodes the proreceptor, a single-chain polypeptide with a molecular mass of 190 kDa that contains one α and one β-subunit. The mature $\alpha_2\beta_2$ heterotetrameric form of the receptor results from dimerization and several posttranslational processing steps, including proteolytic cleavage. An isoform of the receptor lacking 12 amino acids encoded by exon 11 results from alternative mRNA splicing. Insulin receptors lacking exon 11 may have biological properties somewhat different from those containing exon 11 (46), although no significant differences in insulin binding and insulin receptor kinase activity between these two variants were observed (47).

Insulin receptor α-subunits are extracellular structures possessing cysteine-rich domains that serve as insulin-binding sites. Insulin receptor β-subunits have extracellular, transmembrane, and intracellular domains, the latter containing an ATP-binding site and several tyrosine autophosphorylation sites. After insulin binds to the α-subunits, the β-subunits become phosphorylated on tyrosine residues and acquire kinase activity, initiating a cascade of intracellular protein phosphorylation (48, 49). The most important intracellular proteins phosphorylated under the influence of the insulin-receptor tyrosine kinase are the insulin receptor substrates (IRS), several of which have been described (50–58). IRS-1, the first of these to be discovered (2, 59), has a molecular mass of 131 kDa and possesses 14 potential tyrosine phosphorylation sites. IRS-1 appears to be important in insulin receptor function and its variant forms are sometimes associated with diabetes (60, 61). Mice deficient in IRS-2 develop a syndrome resembling type 2 diabetes (62). Some IRS-1 mutations are associated with insulin resistance and hyperinsulinemia (63), and codon 972 polymorphism of the IRS-1 gene is associated with impaired glucose tolerance, PCOS (64), and late onset of type 2 diabetes mellitus (65). IRS-1 binds phosphatidylinositol-3-kinase (PI-3 kinase), a *src* homology-2 (SH2) domain-containing enzyme, activation of which is necessary for the initiation of glucose transport (2, 59, 66–69). In addition to PI-3 kinase activation, mitogen-activated protein kinase (MAPK) is also phosphorylated after insulin receptor binding (2, 49, 59, 70). MAPK activation is thought to be responsible for the growth-promoting effects of insulin (2). MAPK can be activated not only by the insulin receptor, but also by other tyrosine kinase receptors, such as the type I IGF receptor, and receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), as well as G protein-linked receptors (2, 71, 72). The molecular link between the MAPK cascade and the insulin receptor may be p21 Ras, a highly conserved protein involved in cell growth that may be a critical element in growth factor receptor and insulin receptor tyrosine kinase action (2, 49, 59).

Tyrosine kinase activation is believed to be the main signaling mechanism of the insulin receptor (48); it appears to be the earliest postbinding event and is necessary for many, although not all, of insulin's effects, including transmembrane glucose transport (73, 74). Overexpression of tyrosine kinase-deficient insulin receptors in muscle causes insulin resistance in transgenic animals (75). Tyrosine kinase activity is required *in vivo* for phosphorylation of IRS-1 and for PI-3 kinase activation (76).

An alternative signaling pathway for the insulin receptor has also been described. It involves generation of inositol-glycan second messengers at the cell membrane after insulin binding to receptor α-subunits but independently of β-subunit tyrosine kinase activation (77). This alternative pathway for receptor signaling may mediate some of insulin's effects, including stimulation of ovarian steroidogenesis (78–80) (Fig. 1), but the role of this system in propagating the insulin

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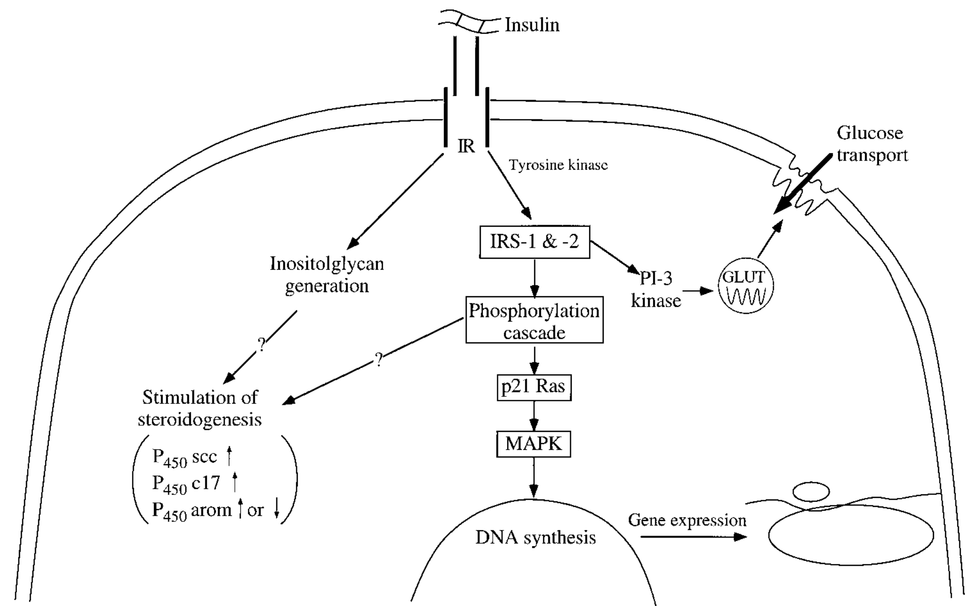

FIG. 1. Insulin receptor, its signaling pathways for glucose transport, and hypothetical mechanisms of stimulation or inhibition of steroidogenesis. The main pathways for the propagation of the insulin signal include the following events: after insulin binds to the insulin receptor α-subunits, the β-subunit tyrosine kinase is activated; IRS-1 and -2 are phosphorylated; PI-3 kinase is activated; GLUT glucose transporters are translocated to the cell membrane, and glucose uptake is stimulated. An alternative signaling system may involve generation of inositolglycans at the cell membrane after insulin binding to its receptor. This inositolglycan signaling system may mediate insulin modulation of steroidogenic enzymes (see text for more details and references).

signal for glucose transport and other insulin effects has not been fully established.

Insulin binding to its receptor results in a plethora of metabolic effects, including stimulation of DNA and protein synthesis, lipogenesis, transmembrane electrolyte transport, and a variety of effects on carbohydrate metabolism, the most important of which is stimulation of transmembrane glucose transport (2). This transport is carried out by a family of glucose transporter proteins (GLUTs) (81) which, in their resting phase, reside in intracellular vesicles. After insulin binds to its receptor, these vesicles are translocated to and fuse with the plasma membrane. The GLUTs are then inserted into the plasma membrane and become functional. Once glucose transport is completed, GLUTs are recycled to intracellular vesicles. Insulin signaling for glucose transporter activation is mediated by PI-3 kinase.

Insulin receptor-like proteins are present in lower organisms that do not produce insulin. For example, in certain species of worms, *daf-2*, a gene similar to that of the insulin receptor, regulates glucose metabolism and longevity (82). Mutation of the insulin receptor in *Drosophila* leads to small ovaries lacking oocytes, and thus sterility (83). Insulin receptor-like molecules are present in mosquito ovaries (84). The existence of these homologous proteins in insects suggests that the growth and regulatory functions of the insulin/IGF receptor family arose before the divergence of insects and vertebrates more than 600 million years ago (83). Conservation of the insulin receptor over this length of time in a variety of organisms indicates its importance for their survival. Indeed, mice with a genetic knockout of the insulin receptor die in the neonatal period (85).

B. Presence of insulin and insulin receptor in the ovary

Circulating insulin levels in the peripheral blood of normal women are approximately 10 μU/ml in the fasting state and up to 50 μU/ml within 1 h after an oral glucose load. In obese women, these levels are somewhat higher, averaging approximately 15 μU/ml in the fasting state and up to 60 μU/ml after a glucose load. In insulin-resistant hyperinsulinemic states such as PCOS or the early stages of type 2 diabetes mellitus, serum insulin levels range from 20–35 μU/ml in the fasting state to 120–180 μU/ml after a glucose load (9, 86). In patients with syndromes of extreme insulin resistance, circulating insulin levels may be as high as 200 μU/ml in the fasting state and up to 1400–2000 μU/ml after a glucose load (9).

Ovarian follicular fluid (FF) insulin concentrations range from less than 2 μU/ml to 65 μU/ml, with a mean value of approximately 16 μU/ml (87). These do not correlate with plasma insulin or FF estradiol (E_2) or androstenedione (A) concentrations, but do correlate directly with those of progesterone (P) (87). Insulin likely reaches FF from the circulation by transudation. To our knowledge, intrafollicular concentrations of insulin have not been reported in women with insulin resistance with or without ovulatory dysfunction.

Both in humans and in animal models, insulin receptors are widely distributed throughout all ovarian compartments, including granulosa, thecal, and stromal tissues (3, 11, 12, 88–91) (Table 2). Ovarian insulin receptors have the same heterotetrameric $\alpha_2 \beta_2$ structure as insulin receptors in other organs. They possess tyrosine kinase activity (12) and may stimulate the generation of inositolglycans (79).

The regulation of insulin receptor expression in the human ovary has been investigated (92, 93). As in other organs, insulin itself plays a major role in this process: *in vitro*, insulin exposure leads to receptor down-regulation, followed by a return to normal receptor number approximately 4 h after insulin exposure ends (92). *In vivo*, down-regulation of ovarian insulin receptors by insulin has been observed in rats with experimentally induced hyperinsulinemia (94). In postmenopausal women, *in vivo* studies have demonstrated a positive correlation between insulin receptor number on circulating white cells and in the ovary (93). This relationship was not found in premenopausal women. Since insulin is the

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TABLE 2. Expression of IGFs, IGFBPs, IGFBP proteases, type I and type II IGF receptors, and insulin receptors in the human ovary[a]

		IGF-I	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-2 protease	IGFBP-3 protease	IGFBP-4 protease	Type I IGF-R	Type II IGF-R	Insulin receptor
Early antral follicles (3–5 mm)	O	–	+									2+		2+
	G	–/–	2+	–/–	3+/4+	–/–	2+	1+				3+/3+	3+	1+
	T	2+/–	2+	–/–	3+/4+	2+	4+/	2+				–/2+	3+	1+
	S	–/–		–/–		–/–	4+/4+	4+/4+				–	–	1+
	V			–/–			4+							
Late antral follicles (7–20 mm)	O	–	2+									4+		2+
	G	–/–	4+/4+	4+/	–/4+	3+/–	–/4+	–/–4+*cu*	+	+	+++	4+/4+	4+	2+
	T	–/–	–	–/–	4+/4+	3+/	2+/2+	2+	+	+	+++	–/2+	3+	2+
	S	–/–		–/–	–	–/–	4+/3+	4+/4+	+	+	+++	–	–	1+
	V				/4+	/4+	/4+							
Corpus luteum (and granulosa luteal cells)	G	–	4+/4+	4+/						+	+++	–/[b]	4+/[b]	2–3+
	T	–		–/–						+	+++		3+	
	S	–		–/–						+	+++	–	–	weak
	V					/4+								

[a] [Data are from Refs. 88,90,91,344,437,458, and 514.] Since there are discrepancies between the groups using *in situ* hybridization, these results are reported in the format of Ref. 88/Ref. 344. Data are presented as strongly positive (4+) to weakly positive (1+). If no number appears, the data were not reported. IGF-R, IGF receptor; O, oocyte; G, granulosa; T, theca; S, stroma; V, vascular endothelium; *cu*, cumulus.

[b] Type I IGF receptor mRNA expression present in granulosa-luteal cells (90).

major regulator of receptor number on peripheral leukocytes, these observations suggest, albeit without direct evidence, that insulin is the major regulator of ovarian receptors in postmenopausal women. In premenopausal women, however, other circulating factors such as gonadotropins or sex steroids, or locally produced autocrine regulators such as IGFs and IGFBPs, may be involved in insulin receptor regulation. These factors may account for the observation that in premenopausal women with PCOS and other hyperinsulinemic states, ovarian insulin receptor expression is preserved (88, 89, 95) and that the insulin receptor may mediate some of the ovarian effects of insulin despite the presence of peripheral insulin resistance (9, 79, 96, 97).

Insulin-induced hyperandrogenism is unlikely to result from an action of insulin through its own receptor, however, in disorders in which receptor expression or availability is significantly compromised, such as the type A syndrome of insulin resistance and acanthosis nigricans, caused by insulin receptor mutations, or the type B syndrome, associated with antiinsulin receptor antibodies (6, 7). In the latter two conditions, insulin receptors likely function as inefficiently in the ovary as in other organs, and another receptor, such as the type I IGF receptor, is more likely to mediate the effects of hyperinsulinemia in the ovary (9).

C. Insulin action and the ovary

Numerous actions of insulin on the ovary have been demonstrated both *in vitro* (Table 3) and *in vivo* (Tables 3 and 4), with no significant differences between humans and other species (3).

1. Effects on steroidogenesis.

a. In vitro studies. In vitro, insulin stimulates ovarian steroidogenesis by both granulosa and thecal cells, increasing production of androgens, estrogens, and progesterone (3, 10, 96–101). In some studies, the concentration of insulin required to achieve a stimulatory effect is supraphysiological (3, 10), suggesting that insulin may be acting through the type I IGF receptor. Several lines of evidence, however, suggest that insulin receptors mediate the stimulation of steroidogenesis by insulin. Willis and Franks (97) demonstrated that insulin-stimulated steroid production by granulosa cells obtained from both normal women and those with PCOS could be inhibited by antiinsulin receptor antibodies, but not by antibodies against the type I IGF receptor. Nestler *et al.* (79) recently demonstrated in cultured thecal cells obtained from women with PCOS that insulin stimulation of testosterone (T) production could not be inhibited by an antibody against the type I IGF receptor, suggesting that this effect of insulin was also mediated by the insulin receptor. Since circulating levels of insulin rarely are high enough to produce significant

TABLE 3. A summary of insulin effects related to ovarian function

Effect	Organ
Directly stimulates steroidogenesis	Ovary
Acts synergistically with LH and FSH to stimulate steroidogenesis	Ovary
Stimulates 17 α-hydroxylase	Ovary
Stimulates or inhibits aromatase	Ovary, adipose tissue
Up-regulates LH receptors	Ovary
Promotes ovarian growth and cyst formation synergistically with LH/hCG	Ovary
Down-regulates insulin receptors	Ovary
Up-regulates type I IGF receptors or hybrid insulin/type I IGF receptors	Ovary
Inhibits IGFBP-1 production	Ovary, liver
Potentiates the effect of GnRH on LH and FSH	Hypothalamus/pituitary
Inhibits SHBG production	Liver

See text for details and references.

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binding to the type I IGF receptor, the actions of insulin on the ovary are likely mediated mainly by the insulin receptor.

At this time, there is only limited knowledge about the specific effects of insulin on ovarian steroidogenic enzymes. A stimulatory effect of insulin on aromatase has been suggested by some studies of animal and human ovarian cells *in vitro* (102–105), but one study (106) failed to confirm this finding. 17α-Hydroxylase activity appears to be stimulated by insulin (29, 107–109), but a recent study of 28 women with PCOS and 18 normal controls found no correlation between insulin levels and 17-hydroxyprogesterone (17-OHP) levels after treatment with GnRH agonist (GnRHa) (110). Insulin increases P_{450} side chain cleavage (scc) enzyme mRNA in porcine granulosa cells (111) and P_{450}scc activity in goldfish follicles (112). A similar effect could not be demonstrated, however, in a human ovarian thecal-like tumor line (101). In the latter study, insulin had no effect on the enzyme activity or mRNA concentration of 17α-hydroxylase/17,20-lyase (P_{450}c17) or 3β-hydroxysteroid dehydrogenase (HSD), but forskolin stimulation of 3β-HSD mRNA was enhanced by insulin. In human luteinized granulosa cells, 3β-HSD expression was found to be stimulated by insulin (106).

b. In vivo studies (Table 4). It has not been consistently demonstrated that insulin stimulates ovarian steroidogenesis *in vivo* (113). Several studies have examined the *in vivo* effects of insulin on aromatase. In rats with experimental hyperinsulinemia, an increased estrone (E_1) to A ratio was demonstrated, consistent with a stimulatory effect of insulin on ovarian or peripheral aromatase (94). In women, an insulin infusion study has suggested a similar effect (114), and in hyperinsulinemic women with PCOS, an increased E_2/A ratio was seen after gonadotropin stimulation, compared with normoinsulinemic women with PCOS (115). Relatively insulin-deficient women with type 2 diabetes show reduced aromatase activity (116). The increase in circulating A level observed during insulin infusions in women (117, 118), on the other hand, suggests that insulin may inhibit aromatase. In short, it remains unclear whether or how insulin regulates aromatase *in vivo*.

The effect of insulin on ovarian androgen production in women has been extensively studied (Tables 3 and 4). In PCOS, a positive correlation has been reported between insulin and T or A levels (119–122) in several studies, while more recent studies (123–127) failed to find such a relationship. In insulin infusion studies that maintained hyperinsulinemia for several hours, a stimulatory effect of insulin on ovarian androgen production has not been consistently found. Stuart and associates (117, 118, 128) demonstrated elevation of A and dehydroepiandrosterone (DHEA) in normal lean and obese women and in women with insulin resistance and acanthosis nigricans during a euglycemic, hyperinsulinemic clamp study. Micic *et al.* (129) demonstrated an increase of T in patients with PCOS during a 4.5-h insulin infusion. On the contrary, Diamond *et al.* (130) could demonstrate no change in total or free T or in A during either insulin or glucose infusion in normal women. Similarly, Nestler *et al.* (131) could not demonstrate a rise in T in normal women during insulin infusion. Dunaif and Graf (114) examined gonadotropin and sex hormone levels basally and during insulin infusion in normal and PCOS women. No effect on gonadotropins was demonstrated; E_2 levels rose in response to insulin in normal women. In PCOS women, A levels increased, but T, free T, and dihydrotestosterone (DHT) levels declined.

Another group of studies has examined the effects of food intake or oral or intravenous administration of glucose on circulating androgen concentrations. In normal women, Parra *et al.* (132) found an increase in free T and no change in A after breakfast, but a decline of free T after an oral glucose load. Elkind-Hirsch *et al.* (133) failed to demonstrate a rise of either T or A during a tolbutamide-enhanced intravenous glucose tolerance test (IVGTT). Smith *et al.* (134) found a positive correlation between insulin responses and A, T, and DHT levels during oral glucose tolerance testing (OGTT) in hyperandrogenic and normal women, but Tiitinen *et al.* (135) demonstrated no significant change in T or A in women with PCOS or weight-matched normal controls after an oral glucose load and Tropeano *et al.* (136) demonstrated a decline of T, A, and DHEA during an OGTT. On occasion, both a stimulatory response and the lack of it have been observed in the same study. For example, Anttila *et al.* (137) reported a tendency to increased serum T levels during OGTT mainly in a subgroup of PCOS patients with both hyperinsulinemia and elevated LH levels; most PCOS patients, however, showed a decline in T. Fox *et al.* (138) found that serum androgens declined in PCOS patients during OGTT, but A rose during a 2-h intravenous insulin infusion in obese controls. Since a decline of serum T in the course of a 3- or 4-h OGTT may be attributed to diurnal variations of T, the lack of an increase of T under these conditions argues against a significant *acute* stimulatory or inhibitory effect of insulin on ovarian androgen production *in vivo*.

While studies that raise circulating insulin concentration have produced variable effects on serum androgen levels, studies in which insulin levels were reduced have consistently demonstrated a decline in serum androgen levels in insulin-resistant hyperandrogenic women (139, 140) (see *Section VI.A*). Whether insulin levels are lowered with diazoxide (30, 141), octreotide (34, 142), metformin (29, 31, 108, 143–146), troglitazone (35, 36), or through weight loss (147–156), a decline in serum androgen levels is usually found and ovulatory function improves (Table 4). In contrast to the studies in which insulin levels were elevated acutely for several hours, the effect of the reduction of circulating insulin can be studied over many weeks. If insulin-induced stimulation of ovarian steroidogenesis requires a prolonged exposure to excess circulating insulin, the latter group of studies is more likely to be able to demonstrate, albeit indirectly, a stimulatory effect of insulin on circulating steroids. A confounding factor in some of these studies is a decline in circulating LH, which may be responsible, at least in part, for the reduced androgen secretion (157).

In summary, it appears that insulin may have stimulatory or inhibitory effects on ovarian steroidogenic enzymes, but the responses of specific enzymes may vary with cell type and possibly among species. Further studies are needed on the effects of insulin on steroidogenic enzymes in the ovaries both *in vitro* and *in vivo.*

2. Interactions with gonadotropins. Acting at the ovarian level, insulin appears to potentiate the steroidogenic response to gonadotropins, both *in vitro* and *in vivo* (96, 102, 157–163). In

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TABLE 4. Selected *in vivo* studies of the effect of insulin on circulating ovarian androgens, SHBG and LH

		Ref	Correlative studies	
	Burghen *et al.*, 1980	119	PCOS and control, obese	I positively correlated with T, A
	Chang *et al.*, 1983	120	PCOS, nonobese	I positively correlated with T, A
	Pasquali *et al.*, 1983	121	PCOS, obese and nonobese	I positively correlated with A
	Elkind-Hirsch *et al.*, 1991	133	PCOS, obese and nonobese; nonobese controls	I positively correlated with T
	Anttila *et al.*, 1991	124	PCOS without acanthosis nigricans, obese and nonobese	I did not correlate with T or A
	Toscano *et al.*, 1992	123	Hirsute women, with and without PCOS, obese and non-obese	I did not correlate with T
	Buyalos *et al.*, 1993	125	PCOS, obese and nonobese	Basal and integrated I on OGTT did not correlate with T or A
			Studies in which circulating insulin levels were raised	
Insulin infusion:	Nestler *et al.*, 1987	131	Nonobese normal women; one obese woman with IR/HA	No change or ↓T in normals; no change in T in IR/HA;
	Stuart *et al.*, 1987	118	Normal obese and nonobese women; obese women with IR/HA;	↑A in all groups
	Micic *et al.*, 1988	129	PCOS, obese	↑T
	Dunaif and Graf, 1989	114	PCOS with IR, most obese; obese controls	PCOS: ↑A, ↓T, ↓fT, ↓DHT Normals: A,T,fT,DHT unchanged
	Stuart and Nagamani, 1990	128	Normal women and women postoophorectomy	↑A in both groups; no change in T
	Fox *et al.*, 1993	138	Normal and PCOS women, obese and nonobese	↑A in normal obese; T unchanged in all groups
	Diamond *et al.*, 1991	130	Normal, nonobese women	No effects on T, fT, or A
IVGTT + tolbutamide:	Elkind-Hirsch *et al.*, 1991	133	PCOS, obese and nonobese; nonobese controls	No change of A or T in either group
OGTT:	Smith *et al.*, 1987	134	Normal nonobese and HA obese women	I positively correlated with A, T, DHT in both groups
	Tiitinen *et al.*, 1990	135	Obese and nonobese PCOS; nonobese controls	No significant effect on A or T in either group
	Anttila *et al.*, 1993	137	Normal and PCOS, obese and nonobese	↓T, ↓A in PCOS; ↓T in normals
	Fox *et al.*, 1993	138	Normal and PCOS, obese and nonobese	↓T, ↓A in all groups except nonobese normal; ↑T in nonobese normal
	Tropeano *et al.*, 1994	136	Normal and PCOS, obese and nonobese	↓T, ↓A in both groups; no correlation between I and T
	Parra *et al.*, 1995	132	Normal women	↓fT after OGTT, ↑fT after breakfast
			Studies in which circulating insulin levels were lowered	
Diazoxide:	Nestler *et al.*, 1989	30	Obese PCOS	↓T, ↓fT, ↓A/E; A and LH unchanged
	Krassas *et al.*, 1998	141	PCOS, obese and nonobese	↓fT, ↓A, ↑SHBG; LH unchanged
Octreotide:	Prelevic *et al.*, 1992	34	PCOS	↓T, ↓A, ↓LH
	Fulghesu *et al.*, 1995	142	PCOS	↓T, ↓A, ↓LH only if hyperinsulinemic

granulosa cells, this effect may be mediated by an increase in LH receptor number, since insulin in concert with FSH increases ovarian LH-binding capacity (13, 164). In addition, insulin may act on the pituitary to increase gonadotrope sensitivity to GnRH. Evidence for this effect comes both from *in vitro* studies (165, 166) and indirectly from studies in insulin-resistant patients treated with insulin sensitizers, in whom circulating LH declined concomitantly with insulin (29, 31, 35, 108). On the other hand, in rats with experimental hyperinsulinemia maintained over six 4-day estrous cycles, the response of gonadotropins to GnRH did not differ from that of controls (94). In normally cycling women, increasing body mass index (BMI) did not have an effect on gonadotropin secretion and in women with PCOS BMI and LH levels were inversely related (167–169), while gonadotropin responsiveness to GnRH did not change after insulin infusion (114). In summary, it remains unclear whether hyperinsulinemia significantly enhances gonadotrope responsiveness to GnRH *in vivo*, as it does *in vitro*.

3. Effects on ovarian growth and cyst formation. In a rat model, a synergistic interaction between LH/hCG and insulin on the ovary can be demonstrated directly during experimentally induced hyperinsulinemia, which enhances hCG-induced ovarian growth and cyst formation (28, 170) (Fig. 2). This synergistic action of insulin with LH/hCG is seen regardless

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TABLE 4. Continued

Studies in which circulating insulin levels were lowered (continued)				
Weight loss:	Kopelman *et al.*, 1981	148	Obese, HA	↓T, ↓A, ↑SHBG
	Bates and Whitworth, 1982	150	Obese PCOS	↓T, ↓A
	Harlass *et al.*, 1984	149	Obese with irregular menses	↓T, ↓LH, ↑SHBG
	Pasquali *et al.*, 1989	151	Obese, HA	↓T, ↓LH
	Kiddy *et al.*, 1992	147	Obese PCOS	↓fT, ↑SHBG; T unchanged
	Holte *et al.*, 1995	155	Obese PCOS	↓T, ↑SHBG; A and LH unchanged
	Guzick *et al.*, 1994	152	Obese PCOS	↓fT, ↑SHBG; LH and T unchanged
Metformin:	Crave *et al.*, 1995	153	Obese, hirsute	↓fT, ↓A, ↑SHBG, T unchanged with weight loss; no additional effect of metformin
	Velazquez *et al.*, 1994	144	Obese PCOS	↓T, ↓fT, ↓A, ↓LH, ↑SHBG
	Nestler and Jakubowicz, 1996	29	Obese PCOS	↓fT, ↓17-OHP, ↓LH, ↑SHBG
	Nestler and Jakubowicz, 1997	108	Nonobese PCOS	↓T, ↓fT, ↓A, ↓LH, ↑SHBG
	Diamanti-Kandarakis *et al.*, 1998	145	Obese PCOS	↓fT, ↓A, ↑SHBG; T unchanged
	Morin-Papunen *et al.*, 1998	146	Obese PCOS	↓fT; T, SHBG, LH unchanged
Troglitazone:	Dunaif *et al.*, 1996	35	Obese PCOS	↓fT, ↓A, ↓LH, ↑SHBG
	Ehrmann *et al.*, 1997	36	Obese PCOS	↓T, ↓fT, ↓A, ↑SHBG; LH unchanged

I, Insulin; fT, free testosterone; A, androstenedione; DHT, dihydrotestosterone; LH, luteinizing hormone; SHBG, sex hormone binding globulin; IR, insulin resistance; HA, hyperandrogenism (hyperandrogenic).

of cotreatment with a GnRH antagonist, suggesting that the growth- and cyst-promoting effects of insulin are exerted directly on the ovary. Indeed, insulin can stimulate proliferation of both human and rat theca-interstitial cells *in vitro* (171–173). In humans, the ability of high insulin levels to stimulate ovarian growth *in vivo* has been suggested by a case report of a patient with the type B syndrome of insulin resistance, whose sonographically determined ovarian volume doubled during a prolonged insulin infusion (174). Furthermore, in women with PCOS, circulating insulin levels are correlated with ovarian volume (175, 176), and after gonadotropin stimulation, the increase in ovarian dimensions observed in hyperinsulinemic PCOS is greater than in normoinsulinemic PCOS (115).

4. Effects on sex hormone-binding globulin (SHBG) production. Closely linked to the steroidogenic effects of insulin is its inhibitory effect on hepatic SHBG production, which has been shown both *in vitro* and *in vivo* (177–180). In fact, SHBG levels may be useful for screening individuals for insulin resistance, since they correlate negatively with circulating insulin levels (181–184). An increase in circulating SHBG, as may be seen in women with PCOS given insulin sensitizers (see *Section VI.A.3*) (29, 31, 35), may lead to decreased circulating levels of free steroid hormones, including free T. Suppression of SHBG production may be largely responsible for hyperandrogenism in some patients with hyperinsulinemic insulin-resistant states.

5. Effects on IGFBP-1 production. Another protein under the regulatory control of insulin is IGFBP-1. Insulin and BMI are the major determinants of circulating IGFBP-1 levels in both obesity (185–187) and PCOS (183, 188–192). Insulin inhibits IGFBP-1 production in the liver (193–198), thereby reducing circulating IGFBP-1 levels. Insulin also inhibits IGFBP-1 production in ovarian granulosa cells (see *Section IV.B*), acting through its own receptor (199). A detailed discussion of the role of IGFBPs in ovarian function and their regulation in the ovary is presented in *Section IV.D*.

6. Ovulation in diabetes mellitus and in states of extreme insulin resistance. Insulin and IGFs have been shown to suppress apoptosis in ovarian follicles, thus reducing rates of their atresia (200, 201). A variety of clinical and experimental observations in patients with type 1 and type 2 diabetes mellitus and states of extreme insulin resistance suggest that insulin may be involved, either directly or indirectly, in the process of ovulation (3, 9, 202).

Insulin deficiency in type 1 diabetes has been associated with disordered ovulation (3, 202). In rats, streptozotocin-induced diabetes is associated with cessation of ovulatory cycles, which can be restored with insulin treatment (203). In mice with alloxan-induced diabetes, a similar reduction in ovulation rate has been reported (204). While the current availability of insulin therapy does not allow observation of a similar phenomenon in human type 1 diabetes, in the preinsulin era, girls who developed diabetes prepubertally failed to enter puberty (3, 4). It is difficult to determine whether it was insulin deficiency itself, the state of chronic diabetic ketoacidosis, the starvation diets used for treatment, or the dramatic weight loss that caused the failure of pubertal development in these girls. In patients with type 1 diabetes treated with insulin, the hypothalamic-pituitary-gonadal axis appears to be relatively hypoactive, mainly because of failure of the GnRH pulse generator (205, 206); low serum sex hormone levels, including low luteal-phase P levels, have been described (207, 208). Even with insulin treatment, up to

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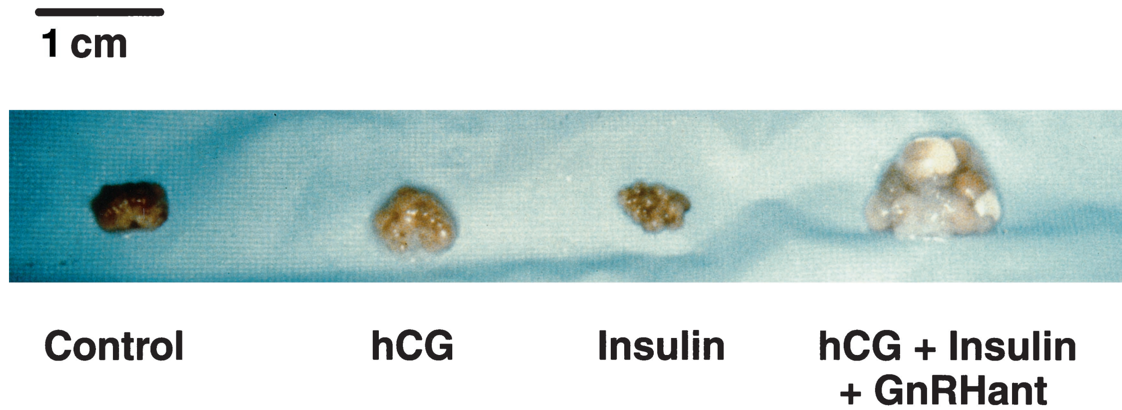

FIG. 2. The effects of 23 days of daily injections of normal saline (control), hCG, insulin, or insulin plus hCG and GnRHant on gross ovarian morphology in rats. Female Sprague-Dawley rats were randomized into the following treatment groups: vehicle; high-fat diet (to control for the effects of weight gain); insulin; hCG; GnRH antagonist (to control for possible central effects of insulin *vs*. direct effects on the ovary); GnRHant and HCG; insulin and GnRHant; insulin and hCG; insulin, hCG, and GnRHant. Ovarian morphology in the group treated with insulin and hCG (not shown) did not differ from that seen in the group treated with insulin, hCG, and GnRHant (shown above). [Reproduced with permission from L. Poretsky *et al.*: *Metabolism* 41:903–910, 1992 (170). ©W. B. Saunders Co.]

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one third of young women with type 1 diabetes may experience delayed menarche and oligomenorrhea of hypothalamic origin (205).

Hyperinsulinemia resulting from exogenous insulin administration is often present in treated patients with type 1 diabetes. If such patients gain excessive weight, their LH:FSH ratio increases, SHBG levels decrease, and more than 70% develop polycystic ovaries (209); the response of 17-OHP to GnRHa in oligomenorrheic diabetic adolescents is exaggerated, resembling the response reported in insulin-resistant patients with PCOS (29, 108, 210). Some patients with type 2 diabetes have mildly elevated androgen levels or increased androgen responses to GnRH stimulation (116, 202) as well as reduced SHBG levels (211), particularly in the early, hyperinsulinemic stage of the disease (116, 212). It should be noted that hyperinsulinemia in patients with diabetes is relatively mild, compared with that seen in patients with syndromes of extreme insulin resistance, and that significant hyperandrogenism is not characteristic of women with either type 1 or type 2 diabetes (9).

Hyperandrogenism and polycystic ovaries or ovarian hyperthecosis are commonly found in states of extreme insulin resistance (9, 140, 213). These conditions are sometimes caused by mutations of the insulin receptor gene (214–216) and include the type A syndrome (6), leprechaunism (9, 217, 218), Rabson-Mendenhall syndrome (9, 215), and syndromes characterized by defective insulin receptor signaling (74, 219, 220). Premenopausal patients with the type B syndrome (insulin resistance and acanthosis nigricans associated with the presence of antiinsulin receptor antibodies) also exhibit hyperandrogenism (7, 8).

Although there is evidence that hyperinsulinemia contributes to the development of hyperandrogenism, not all clinical conditions associated with hyperinsulinemia lead to ovarian androgen overproduction. For example, most women with type 1 diabetes, who are often hyperinsulinemic because of exogenous insulin administration but usually do not exhibit significant insulin resistance, do not become hyperandrogenic, but rather exhibit hypothalamic-pituitary-ovarian axis hypofunction. It is not clear why hyperinsulinemia developing in the setting of insulin resistance, rather than any form of hyperinsulinemia, is associated with ovarian hyperandrogenism, particularly since correction of hyperinsulinemia without correction of insulin resistance may improve ovarian function (38, 221–223).

Dissecting the effects of hyperinsulinemia from those of insulin resistance is difficult (224, 225). One can postulate, however, that because the postbinding insulin receptor pathways may diverge (2, 9, 226), in conditions characterized by hyperinsulinemia without primary insulin resistance all insulin receptor-signaling pathways are significantly downregulated, whereas when hyperinsulinemia is caused by insulin resistance, only some of these pathways (*e.g.*, glucose transport) may be deficient, while others may be hyperstimulated (9, 227, 228). Thus, if hyperinsulinemia promotes androgen production by activating insulin-signaling pathway(s) distinct from those involved in glucose transport, hyperandrogenism would be more likely to develop in the setting of insulin resistance and compensatory hyperinsulinemia.

7. Interactions of insulin with leptin; leptin-mediated effects on ovulation. New insights into the relationship between weight and ovulation and the role that insulin may play in modifying this relationship emerged with the discovery and characterization of leptin. Leptin is a 16-kDa protein produced by adipose cells (229–233). Circulating leptin levels are stimulated by estrogen and inhibited by androgens (234–236) and are directly proportional to adipose tissue mass (236–241). Leptin regulates body weight by binding to specific receptors in the hypothalamus and thus decreasing food intake (242–244). Leptin is encoded by the *ob* gene, which is defective in genetically obese *ob/ob* mice (229, 231, 237, 245). These animals are also insulin resistant and infertile. Replacement of leptin in *ob/ob* mice produces weight loss, reverses metabolic abnormalities, and restores ovulation and fertility (246, 247). *Db/db* mice and Zucker fatty rats have a similar phenotype, which results from a genetic abnormality of the leptin receptor (237, 245, 248). A human kindred with an *ob* mutation has been described, in which two prepubertal cousins with

a frameshift mutation in the *ob* gene suffer from massive obesity (249). It is not yet known whether they will develop reproductive abnormalities. Similarly, a mutation of the human leptin receptor gene associated with obesity has been reported (250).

A rise in circulating leptin levels is associated with and precedes puberty (251), and higher circulating leptin levels are associated with a younger age at menarche (252, 253), possibly because leptin serves as a signal for the initiation of an early pubertal gonadotropin-secretory pattern (254–257). A rapid decline of circulating leptin levels is observed during caloric restriction (258) or starvation (244, 259, 260). A decline in leptin may be responsible for the activation of the hypothalamic-pituitary-adrenal axis and the inhibition of the gonadotropic axis observed with stress (261, 262), since these responses can be abolished in animals by leptin administration (233, 263).

Leptin receptors are present in the ovary (264–266). Their functional capacity and their role in both normal and abnormal ovarian function remain to be firmly established since two leptin receptor isoforms exist, one with a full-length and another with a truncated intracellular domain (267). While the action of leptin on gonadotropin secretion is stimulatory, the direct effects of leptin on ovarian steroidogenesis may be either inhibitory or stimulatory (264, 266, 268). For example, leptin inhibits insulin-induced P and E_2 production in bovine granulosa cells (264) and reduces synergism between FSH and IGF-I on E_2 production in rat granulosa cells (268). On the other hand, leptin appears to stimulate ovarian 17α-hydroxylase (265).

Insulin stimulates secretion of leptin by adipocytes (269–272). In addition, by promoting lipogenesis, insulin may increase adipose tissue mass, thereby further enhancing leptin production. However, there is no apparent acute effect of feeding on leptin levels (260, 273, 274) and no correlation between leptin and insulin sensitivity *in vivo* (273). Nevertheless, circulating leptin levels rise with acute massive overfeeding over a 12-h period (275).

Leptin inhibits insulin secretion from isolated pancreatic islets in some studies (276, 277), but stimulates insulin secretion in others, either by a direct stimulatory effect on pancreatic β-cells (278) or because of its inhibitory effect on somatostatin (279). Leptin may affect pancreatic function through the autonomic nervous system (280) and was shown to improve insulin sensitivity in normal rats, reducing glucose and insulin levels (281). When administered intracerebroventricularly, leptin enhanced insulin-stimulated glucose metabolism (282). Leptin has been shown to possess antidiabetic properties in some studies (283, 284), but in other studies it did not affect glucose-stimulated insulin secretion and did not have a significant effect on glucose transport or insulin action in either adipocytes or muscle cells (285, 286). In some circumstances, as, for example, in the setting of obesity, leptin may contribute to the development of insulin resistance and diabetes (287–290).

The above observations point to a complex relationship among insulin, leptin, body weight, ovarian steroidogenesis, and ovulation (Fig. 3). If a certain "threshold" level of leptin is needed to activate the hypothalamic-pituitary-ovarian axis, then a certain mass of adipose tissue must be present for

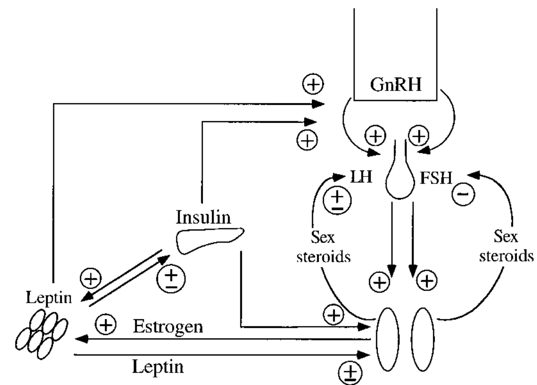

FIG. 3. The relationships among insulin, leptin, pituitary gonadotropins, and ovarian steroidogenesis. Insulin stimulates leptin secretion, enhances pituitary gonadotropin response to GnRH, and promotes ovarian steroidogenesis. Leptin stimulates the hypothalamic-pituitary-gonadal axis at the level of the hypothalamus and/or pituitary; it inhibits ovarian E_2 and P production, but may stimulate androgen production by stimulating 17 α-hydroxylase activity or expression. Leptin and insulin potentiate each other's secretion, although leptin may inhibit insulin secretion under some circumstances. Ovarian sex steroids inhibit FSH production and either inhibit (E_2, T, P) or stimulate (E_1) LH responsiveness to GnRH.

ovulation to occur (291). In states characterized by hypoinsulinemia, such as starvation, weight loss, or untreated type 1 diabetes mellitus, amenorrhea may develop (292, 293), possibly because of a decline in circulating leptin (294) and a resultant deactivation of the hypothalamic-pituitary-ovarian axis (233, 293, 295). Thus, insulin deficiency may contribute to abnormalities of ovulatory function either directly, by affecting gonadotropins or the ovaries, or indirectly, by negatively influencing secretion of leptin. On the other hand, states characterized by insulin excess may be associated with higher circulating levels of leptin. Whether such putative leptin excess would play a role in the development of the hyperandrogenism or anovulation observed in hyperinsulinemic states remains to be determined.

8. Effects of insulin on expression of ovarian type I IGF receptors. In addition to participating, directly or indirectly, in the regulation of ovarian steroidogenesis and insulin receptor number in the ovary, insulin may also affect the expression of ovarian type I IGF receptors. *In vivo* studies in rats demonstrated that experimental hyperinsulinemia, while down-regulating ovarian insulin binding, increased ovarian IGF-I binding (94) (Fig. 4). That this phenomenon may also occur in humans is suggested by the observations of Samoto *et al.* (95) and Nagamani and Stuart (296), who demonstrated that in women with hyperthecosis or PCOS, ovarian type I IGF receptors are up-regulated, while insulin receptors are down-regulated. Pepper and colleagues (297) have reported that ovarian [^{125}I]IGF-I binding in a patient with ovarian hyperthecosis was increased over that found in normal controls (12, 298). Interestingly, an increase in type I IGF receptor expression in PCOS may not be limited to the ovaries: a rise in erythrocyte type I IGF receptors in these patients has also been reported (299). Further, hyperinsulinemia may increase expression of hybrid insulin/type I IGF receptors in a variety of insulin target tissues (300), although this process has not yet been described in the ovary.

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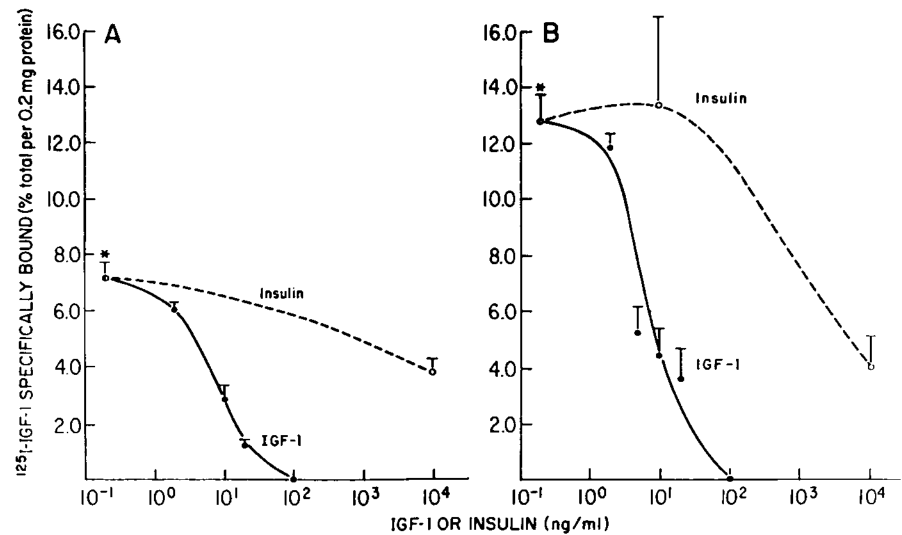

FIG. 4. [^{125}I]IGF-I binding to ovarian homogenates from normal rats (A) and rats with experimentally induced hyperinsulinemia (B). Female Sprague-Dawley rats were treated with either vehicle (A) or insulin for 23 days. [^{125}I]insulin (not shown) and [^{125}I]IGF-I binding to ovarian homogenates was examined. In rats treated with insulin, a doubling of [^{125}I]IGF-I binding was observed, suggesting amplification of the number of type I IGF receptors or hybrid insulin/type I IGF receptors. [Reproduced with permission from L. Poretsky *et al.*: *Endocrinology* 122:581–585, 1988 (94). © The Endocrine Society.]

In addition to up-regulating type I IGF receptors in the ovary, insulin may also increase the cellular pool of p21 Ras (49, 301). Both up-regulation of type I IGF receptors and an increase in the pool of p21 Ras may amplify the effects of IGF-I on steroidogenesis and follicle development. Furthermore, up-regulation of type I IGF receptors may also amplify the effects of IGF-II, the dominant ligand for the type I IGF receptors in human granulosa cells (see *Section III.B*). Finally, up-regulation of type I IGF receptors by insulin may amplify the effects of insulin itself in states of extreme insulin resistance, in which circulating concentrations of insulin are very high and insulin receptors are either genetically defective or blocked by antiinsulin receptor antibodies. Under these circumstances, as discussed previously, insulin may act mainly by binding to the type I IGF receptor via the "specificity spillover" effect (9, 302). Thus, the ability of hyperinsulinemia to up-regulate ovarian type I IGF receptors may contribute to the ovarian growth and stimulation of steroidogenesis by IGF-I, IGF-II, and insulin.

D. Summary

The role of insulin in the ovary may be summarized as follows: 1) Insulin receptors are widely distributed throughout all ovarian compartments. Ovarian insulin receptors have a subunit structure identical to insulin receptors in other organs, possess tyrosine kinase activity, and are capable of stimulating the generation of inositolglycan second messengers. 2) At this time there is no convincing direct *in vivo* evidence that hyperinsulinemia acutely stimulates ovarian steroid production, but there is direct *in vitro* evidence and indirect *in vivo* evidence for a stimulatory effect of insulin on ovarian steroidogenesis. The *in vitro* evidence suggests that the stimulatory effect of insulin on steroidogenesis is mainly mediated by the insulin receptor and may involve the inositolglycan pathway. The *in vivo* evidence is largely derived from experiments in which a reduction in circulating insulin levels produces a decline of circulating androgens and from clinical observations in women with both insulin deficiency and insulin excess. 3) The effects of insulin on ovulation are complex. A threshold level of insulin is likely to be required for the normal function of the hypothalamic-pituitary-ovarian axis, either because of the direct stimulatory effects of insulin on this axis or because of the stimulatory effects of insulin on leptin secretion (both direct, with insulin stimulating adipocyte production of leptin, and indirect, because of insulin-stimulated lipogenesis). Leptin, in turn, participates in the initiation of puberty and activation of the hypothalamic-pituitary-gonadal axis. On the other hand, excessive circulating insulin, particularly in the setting of insulin resistance, may enhance ovarian androgen production and thus may contribute to the development of anovulation. 4) Insulin may amplify its own effects, the effects of IGFs, and those of gonadotropins by up-regulating type I IGF receptors and gonadotropin receptors, as well as by inhibiting production of IGFBP-1, both in the liver and ovary. In the setting of insulin resistance and hyperinsulinemia, therefore, a cycle of events that leads to a self-perpetuating amplification of the ovarian effects of insulin and IGFs can develop (Fig. 5).

In reviewing the literature dealing with the effects of insulin on ovarian function, it is important to distinguish those effects that have been mainly demonstrated *in vitro* or in animal systems, and therefore may contribute only in a limited way to our understanding of normal and abnormal human ovarian physiology, from those that have been clearly demonstrated in women *in vivo*. In our opinion, the only insulin-related effects on ovarian function that have been consistently observed in women *in vivo* are insulin-induced suppression of hepatic SHBG and IGFBP-1 production. The importance of these effects in both normal and pathological conditions still needs to be clarified. The importance for normal and abnormal human ovarian function of the other insulin effects discussed in this section, such as its direct effects on ovarian steroidogenesis, growth, and cyst formation; its effects on the expression of ovarian receptors for insulin, IGF-I, and LH; and its synergistic action with gonadotropins, remains to be established. The reported ovarian effects of insulin *in vitro* and *in vivo* are summarized in Tables 3 and 4.

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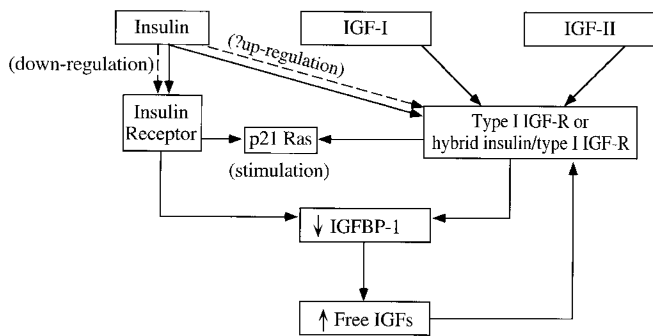

FIG. 5. Hypothetical insulin/IGF self-enhancement mechanisms in the ovary. Hyperinsulinemia, acting through insulin receptors, type I IGF receptors, or possibly through hybrid insulin/type I IGF receptors increases the number of type I IGF receptors and/or hybrid insulin/IGF receptors and increases cellular pool of p21 Ras, which may be responsible for the mitogenic effects of insulin or of IGFs. Hyperinsulinemia also inhibits IGFBP-1 production, leading to a further increase in bioavailable IGFs. Thus, hyperinsulinemia may lead to a self-perpetuating cycle of events resulting in the exaggeration of the ovarian effects of both insulin and IGFs, leading to ovarian enlargement and excessive androgen production (please see the text for details and references). *Solid arrow*, action via a receptor; *broken arrow*, regulation of a receptor.

III. IGFs and Their Receptors

A. IGF peptides and receptors

1. IGF-I. IGF-I is a 70 amino-acid, single-chain polypeptide that shares significant sequence homology with IGF-II, proinsulin, and relaxin. The human IGF-I gene is located on chromosome 12. The major source of circulating IGF-I is the liver, but IGF-I is widely expressed in most tissues, especially during postnatal development (303). IGF-I was first known as somatomedin C and identified as a mediator of GH action (304). GH rapidly activates IGF-I gene transcription and also regulates changes in chromatin structure within the IGF-I gene, delineating a target within the chromatin for GH action (305). In addition to GH, other activators of IGF gene transcription include estradiol, experimental diabetes, and angiotensin II (306). Null mutants for IGF-I are severely growth restricted *in utero* but are fertile (307, 308).

2. IGF-II. IGF-II is a 7.5-kDa, 67-amino acid, single-chain polypeptide that is approximately 70% homologous with IGF-I and 50% homologous with proinsulin (14, 309–312). The human IGF-II gene is located on chromosome 11, contiguous with the insulin gene. Pre-pro-IGF-II, the precursor of IGF-II, is a 22-kDa protein. Inactivation of the IGF-II gene in animals (308, 313) produces growth-deficient but fertile and otherwise normal individuals. IGF-II is highly expressed in fetal tissues and tumors, as well as in normal adult tissues. IGF-II can bind to type I and type II IGF receptors (see below), as well as to the insulin receptor (302, 314).

3. Type I IGF receptor. The type I IGF receptor precursor protein consists of 1367 amino acids, comprising both the α- and β-subunits of the receptor. The human type I IGF receptor gene is located on chromosome 15. The mature type I IGF receptor protein is a heterotetramer consisting of two α- and two β-subunits and is highly homologous with the insulin receptor (315, 316). The cysteine-rich regions of the α-subunits of the insulin receptor and type I IGF receptor are 64–67% homologous, whereas the tyrosine kinase domains of the β-subunits are 84% homologous. In addition to IGF-I, the type I IGF receptor can also bind IGF-II and insulin, although with somewhat lower affinity. In addition to binding IGF-I, IGF-II, and insulin, the type I IGF receptor has also been reported to interact with IGFBPs (317), but the significance of this finding remains to be determined. Type I IGF receptor postbinding events, similar to those of the insulin receptor, include tyrosine phosphorylation of receptor β-subunits and IRS proteins, interactions with PI-3 kinase, and activation of MAPK (69, 315, 318, 319). Type I IGF receptor knockout mice weigh 45% of normal at birth and die immediately afterward (320). Patients with a deletion of the distal arm of chromosome 15 lack one copy of the IGF-I receptor gene and exhibit both intrauterine and postnatal growth restriction (321, 322).

4. Hybrid insulin/type I IGF receptors. Hybrid receptors that combine an α/β insulin hemireceptor and an α/β type I IGF hemireceptor have been reported in a variety of tissues, although not in the ovary (41, 323). These receptors can form in tissues coexpressing both insulin and type I IGF receptors, theoretically including the ovary. Hybrid receptors have properties similar to type I IGF receptors, binding IGF-I with high affinity and insulin with lower affinity. Interestingly, in situations that are characterized by insulin receptor down-regulation, the number of hybrid insulin/type I IGF receptors tends to increase (228).

5. Type II IGF receptor. The type II IGF receptor is identical to the mannose-6-phosphate (Man-6-P) receptor (309, 324–326). The gene for the type II IGF receptor is located on the long arm of chromosome 6. This receptor targets Man-6-P-containing enzymes from the Golgi apparatus to the lysosomes and also mediates the rapid internalization of IGF-II (309). The receptor is a single-chain polypeptide of approximately 300 kDa with a large extracellular domain containing IGF-II binding sites (325, 327). The cytoplasmic domain is very short and includes tyrosine, threonine, and serine phosphorylation sites. Type II IGF receptor knockout mice exhibit elevated IGF-II levels and die *in utero* (328, 329). Interestingly, if the IGF-II gene is knocked out at the same time, about 50% of the fetuses survive to birth (328). Type I/type II IGF receptor double-knockout mice differ from normal controls only in their patterns of growth (328). These observations, taken together, suggest that excessive activation of the type I IGF receptor by IGF-II may be lethal *in utero*.

The type II IGF receptor can be released from the cell membrane into the circulation. This mechanism may be principally responsible for its loss from the cell surface (330–333). The circulating form of the IGF-II receptor retains its affinity for IGF-II (325, 334) and may participate in the local modulation of organ size *in vivo*. For example, overexpression of the soluble IGF-II/Man-6-P receptor in transgenic mice can significantly decrease the weight of their alimentary canal (335).

Although the type II IGF/Man-6-P receptor is important for IGF-II internalization and degradation, it is unclear whether this receptor actively mediates IGF-II signaling. Ex-

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amples of such signaling have been reported, including stimulation of G-protein activation and of thymidine incorporation into rat hepatocyte DNA (325, 336–338). In most instances, however, the metabolic and growth-promoting actions of IGF-II appear to be mediated by the type I IGF receptor (339) or the insulin receptor (314). The type II IGF receptor, however, may mediate signals involved in angiogenesis (340) and other processes. Ligands for the type II IGF receptor, in addition to IGF-II and Man-6-P, include β-galactosidase and other lysosomal enzymes, proliferin, renin, latent transforming growth factor (TGF)-β (329), and leukemia-inhibitory factor (341). In the context of these observations, the functions of the type II IGF receptor within the ovary remain to be determined.

B. Expression of IGFs and IGF receptors in the ovary

1. Human and nonhuman primate. Distinctive features of IGF expression in the primate ovary include the predominance of IGF-II and its pattern of localization (Table 2). Other molecules that modulate IGF action, including the IGF receptors, IGFBPs, and IGFBP proteases, are also differentially expressed in the primate ovary (see below). While the majority of studies that examined the ovarian expression of IGFs and that of their receptors were done on human tissue, ovaries from cycling rhesus monkeys reveal similar expression patterns of IGF-I, IGF-II, and type I IGF receptor, and there is strong evidence that IGF-II, aromatase, and IGFBP-4 can be regarded as markers of the dominant follicle in the rhesus ovary (342).

In the human ovary, IGF peptide expression is follicle stage-specific and compartmentalized (Table 2). IGF-I mRNA is barely detectable in the adult ovary and not in the granulosa layer at any stage of follicular development (88, 89, 343). IGF-II mRNA is expressed in the theca and perifollicular vessels of all follicles and in the granulosa cells of some follicles. In small antral follicles, IGF-II mRNA and protein are detectable in both granulosa and theca (88, 89, 343). In atretic antral follicles, on the other hand, IGF-II is minimally expressed by the theca. IGF-II is abundantly expressed and secreted by granulosa cells of preovulatory follicles as well as by granulosa-luteal cells harvested during oocyte retrieval after controlled ovarian hyperstimulation (COH) (88, 90, 344–347). These findings, plus the observations that granulosa cells do not express IGF-II prepubertally, but do so in a subpopulation of adult follicles, and that gonadotropins regulate IGF-II mRNA expression and secretion in human granulosa-luteal cells *in vitro* (344, 345), suggest that ovarian IGF-II gene expression is regulated by gonadotropins.

Follicular fluid (FF) constituents such as IGF peptides are derived from the circulation as well as from intraovarian production. In normally cycling women, FF IGF-I levels are similar in estrogen-dominant and androgen-dominant follicles and do not correlate with follicular size (348). In contrast, FF IGF-II levels are higher in estrogen- compared with androgen-dominant follicles and correlate positively with follicle size, cycle day, and E_2 and negatively with androgen-estrogen (A:E) ratio (348). In normally cycling women, simultaneous measurements of IGF-I, IGF-II, and insulin concentrations in ovarian and peripheral venous blood reveal an ovarian gradient only for IGF-II (349), and serum IGF-I and IGF-II levels in normally cycling women do not vary during the menstrual cycle (348). These data collectively suggest that FF IGF-I originates from serum by transudation and that FF IGF-II derives primarily from local production by the granulosa and possibly by the theca, in addition to some contribution from the circulation. After COH, FF IGF-II levels are about 8 times higher than those of IGF-I, and both IGF-I and IGF-II levels are lower than in serum (350–353). In contrast to spontaneous cycles, these levels in COH do not correlate with follicle size, oocyte maturity, or FF E_2. FF IGF-I and IGF-II levels were noted to rise with increasing cycle day 3 serum FSH, an index of ovarian reserve (354).

Normal circulating levels of IGF-I are not a prerequisite for normal ovarian follicular development in women, as evidenced by cases of ovulation and fertility in individuals with Laron-type dwarfism, which results from GH receptor deficiency (GHRD) (355–358). Furthermore, a normal follicular response to injected gonadotropins, leading to ovulation and conception, has been reported in women with GHRD, whose serum GH was markedly elevated and both serum and FF IGF-I barely detectable (355, 356). In such subjects, serum IGF-II levels were about 25% of normal (FF IGF-II was not measured). These clinical observations support the conclusion that IGF-I does not play an important role in the ovulatory process in women.

Both type I and type II IGF receptors are found in the human ovary (88, 298, 343, 359). By *in situ* hybridization, type I IGF receptor mRNA is predominantly expressed by granulosa cells and oocytes, with more intense expression in dominant compared with small antral follicles (88, 343). By this technique, theca and stroma are negative for type I IGF receptors, but stromal receptors with the specificity of the type I IGF receptor have been reported in ligand binding studies (298). Type II IGF receptors are localized to both granulosa and thecal layers, with more intense expression in the granulosa and in dominant, compared with smaller, antral follicles (88). By RT-PCR, both types of receptors were found to be expressed by granulosa, theca, and stroma and to persist upon culture of both granulosa and thecal cells (347).

2. Rodent. In the rat, ovarian IGF-I gene expression and protein production are granulosa specific (360–362); significantly, IGF-I is selectively expressed in the granulosa of only healthy antral follicles, not in atretic or luteinized follicles or in theca-interstitial cells (342, 360, 363, 364). IGF-II mRNA expression is limited to the thecal compartment and blood vessels (342, 362, 363), but the postnatal decline in ovarian IGF-II content (365) argues against a significant role for this peptide in rat ovarian physiology. While type I IGF receptor mRNA is abundantly expressed in granulosa cells (365), the corresponding protein is detected not only in the granulosa but also in the thecal compartment, regardless of the maturational stage or health status of the follicle (363), suggesting that regulation of the receptor is unlikely to play a major role in follicular maturation (366).

The patterns of IGF-I, IGF-II, and type I IGF receptor expression are essentially the same in rat and mouse ovary (342, 364, 367). IGF-I expression increases at the secondary preantral stage and is abundant in healthy follicles through the preovulatory stage. Type I IGF receptor is expressed consti-

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tutively, regardless of follicular developmental stage or health (367). These findings lay the groundwork for studies of ovarian function in transgenic mouse models with deletions of these components (368).

3. Livestock species. Porcine granulosa cells in culture secrete abundant immunoreactive IGF-I, which is increased by FSH, cAMP, GH, EGF, and TGF-α. IGF-I is abundant in porcine FF, especially in large follicles. Its levels increase in response to PMSG and/or GH treatment (369–371). This finding suggests that gonadotropin and GH action on the granulosa cells of the developing porcine follicle is mediated in part by local induction of IGF-I. IGF-II in the porcine ovary is expressed mainly in the theca and is not under gonadotropin or GH regulation (15, 370, 372). FF IGF-II levels decline in response to GH (370, 372, 373). In the sheep ovary, at least four localization studies of IGF-I expression have been published, with divergent findings (374–377). IGF-II is localized to the theca, and its levels in FF are 4-fold greater than those of IGF-I (377, 378). In the cow, IGF-I is produced by the ovary (379, 380), and its levels in FF increased with increasing E_2 concentrations and increasing follicle diameter in some (379, 381–384), but not all (385–387), studies. IGF-II is exclusively expressed in the theca, with greater expression in dominant follicles, compared with subordinate or nonrecruited ones (388).

C. Role of IGFs in ovulatory function and steroidogenesis (Table 5)

1. Human. Studies of the effects of IGFs on human granulosa and thecal cells *in vitro* have primarily employed IGF-I, although as discussed above, the predominant endogenous locally produced ligand *in vivo* is IGF-II. IGF actions on the ovary include augmentation of DNA synthesis and steroidogenesis. IGF-I stimulates DNA synthesis and basal E_2 secretion in granulosa and granulosa-luteal cells and inhibits IGFBP-1 production (199, 389–396). It also synergizes with gonadotropins in augmenting E_2 and P production (393, 397–400). Several studies have been conducted recently of the effects of IGF-II on human ovarian cellular constituents. IGF-II stimulates basal P and E_2 secretion by human granulosa-luteal cells (353, 401). It also stimulates aromatization of androgen precursors (402) and inhibits IGFBP-1 (396) and IGFBP-2 (403) production by these cells. The effect of IGF-II on estradiol production is most pronounced if the cells are preincubated with insulin (402), possibly due to insulin-induced up-regulation of type I IGF receptors, formation of hybrid insulin/IGF-I receptors, or inhibition of IGFBP-1 production. IGF-II also stimulates granulosa-luteal cell DNA synthesis and proliferation *in vitro* (401, 404). In granulosa cells from both unstimulated and gonadotropin-stimulated preovulatory follicles, IGF-I, both alone and in synergy with gonadotropins, stimulates P450 aromatase mRNA expression and activity (405).

IGFs also exert actions on human thecal cells and oocytes. In human thecal monolayer cultures, IGF-I enhances DNA and androgen synthesis (406) and synergizes with LH in A production (100), although *in vivo*, a decline of circulating IGF-I levels after treatment with clomiphene citrate did not lead to a reduction in hyperandrogenism in PCOS (407). IGF-II also increases androgen production by human theca (158). Maturation of immature human oocytes *in vitro* can be augmented by IGF-I (408).

2. Rodent. IGF-I actions in rat granulosa and theca have been extensively reviewed (14, 23, 409, 410). IGF-I acts as a co-gonadotropin with FSH to stimulate granulosa cells to produce E_2 and P, and with LH to stimulate thecal androgen production. IGF-I stimulates LH receptor expression in granulosa and theca (13, 411, 412) and may be required for FSH

TABLE 5. Ovarian actions of IGF-I and IGF-II

Species	Granulosa (granulosa/luteal) cells	Theca cells/explants	Follicles
Human			
	Promotes:	*Promotes:*	*Promotes:*
	Aromatase activity and mRNA	Androstenedione production	?Oocyte maturation
	Basal E_2 and P secretion	Testosterone production	
	FSH-stimulated E_2 and P secretion	DNA synthesis	
	DNA synthesis		
	Cellular proliferation		
	IGFBP-4 proteolysis		
	IGFBP-5 production		
	?IGFBP-2 proteolysis		
	Inhibits:		
	IGFBP-1, IGFBP-2 production		
Rat			
	Promotes:	*Promotes:*	*Promotes:*
	Adenylate cyclase	Androstenedione production	?Ovulatory rupture
	Aromatase activity	P_{450}scc mRNA	
	E_2 secretion	17α-Hydroxylase	*Inhibits:*
	LH receptor synthesis	DNA synthesis	Apoptosis
	Progesterone release	Cellular proliferation	
	Inhibin secretion		
	Proteoglycan synthesis		
	DNA synthesis		
	Inhibits:		
	IGFBP-5 proteolysis		

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receptor expression in granulosa (368); it also stimulates granulosa cell production of inhibin α-subunit and augments the stimulation of this response by FSH (413–415). Stimulation of inhibin-α expression in rat granulosa by FSH requires activation of protein tyrosine kinases by endogenously produced IGF-I, suggesting that IGF-I signaling is obligatory for this response (415). IGF-I also stimulates DNA synthesis in granulosa and theca-interstitial cells (171, 416).

In addition to its role in differentiation and proliferation of granulosa and theca, IGF-I also plays an important role in granulosa survival, since it can inhibit apoptosis (201). Granulosa cell apoptosis, associated with regular cleavage of nuclear DNA by endonuclease, is associated with follicular atresia (417). *In vitro*, this process is suppressed by IGF-I and gonadotropins and enhanced by the presence of IGFBPs (200). In the human ovary apoptosis is characteristic of androgen- but not estrogen-dominant follicles (418), but regulation of apoptosis by IGFs has not yet been demonstrated in human ovarian follicles or cellular components, as it has in the rat (201). To our knowledge, there are no studies examining specific effects of IGF-II in rodent ovaries.

3. Livestock species. In the sow, similar effects of IGFs on granulosa and thecal cell function have been reported as in humans and rodents (419–421). IGF-I stimulates granulosa cell proliferation and synergizes with FSH in granulosa cell differentiation (419). IGF-II enhances the delivery of cholesterol to the P_{450} scc enzyme complex and enhances the functional activity of this first committed step in P biosynthesis (421). In sheep, IGF-I stimulates granulosa cells from small follicles to proliferate and those from larger follicles to produce P (422), an effect likely mediated through the type I IGF receptor (423). In the cow, IGF-I stimulates granulosa and thecal cell proliferation and steroidogenesis (379, 380, 424).

D. Summary

Although both IGF-I and IGF-II have been shown *in vitro* to have multiple ovarian effects in various species, IGF-II appears to be the predominant ovarian IGF in the human. The IGF-II gene is expressed in the human ovary, and the effects of IGF-II appear to be similar to those of IGF-I. The metabolic and growth-related effects of IGF peptides appear to be mediated under most circumstances by type I IGF receptors, which are present in all human ovarian compartments. Their numbers appear to be increased under the influence of insulin, as discussed in *Section II.C*. Type I IGF receptors may mediate the effects of insulin in the ovary in extreme insulin-resistant states with severe hyperinsulinemia. Clarification of the presence and the role of hybrid insulin/type I IGF receptors in the human ovary awaits further studies.

IV. IGF-Binding Proteins (IGFBPs) and Proteases

A. Structural relationships among IGFBPs

The bioavailability and, therefore, the actions of the IGFs are regulated, in part, by a superfamily of homologous proteins, called IGFBPs, that bind IGFs with high affinity. There are six IGFBPs, designated IGFBP-1 through IGFBP-6 (425–427), whose discovery, gene and protein structures, and mechanisms of actions have recently been reviewed (329, 428, 429).

All six IGFBPs have core molecular masses of 23–32 kDa. They are all at least 50% homologous, and for each IGFBP there is roughly 80% homology among species. The amino and carboxy termini are most highly homologous among the different IGFBPs, while the midsequence shows little similarity. The IGFBPs each contain at least 16 conserved cysteines, which are important in determining their conformation. There is also a group of proteins that share limited sequence homology with the IGFBPs and bind IGFs with low affinity. Due to their undefined roles as IGFBPs and limited structural homology to IGFBPs 1–6, they have been called IGFBP-related proteins (IGFBP-rPs) (427, 428). The high-affinity IGFBPs have dissociation constant (K_d) values for the IGFs in the range of 10^{-9} to 10^{-11} mol/liter, compared with 10^{-6} to 10^{-7} mol/liter for the IGFBP-rPs (428).

The genes for human IGFBP-1 and IGFBP-3 are located on chromosome 7, the IGFBP-2 and IGFBP-5 genes are on chromosome 2, the IGFBP-4 gene is located on chromosome 17, and the IGFBP-6 gene is on chromosome 12 (329, 430). IGFBP genes are in close proximity to homeobox (Hox) gene clusters (Hox A–Hox D), with which they appear to have coevolved. Hox genes encode DNA-binding proteins that are transcriptionally regulated by retinoic acid, as are some of the IGFBPs (430). IGFBP-1 and IGFBP-2 both contain the tripeptide motif Arg-Gly-Asp (RGD), which can bind to integrins, and their production and function are related to carbohydrate metabolism and metabolic homeostasis. In contrast, IGFBP-3, and likely the highly homologous IGFBP-5, are primarily involved in growth.

The IGFBPs have several functions, which include 1) to transport the IGFs in the circulation; 2) to regulate efflux of IGFs from the vascular space; 3) to prolong the half-life and metabolic clearance rates of the IGFs; 4) to prevent IGF-induced hypoglycemia; 5) to directly modulate interactions of IGFs with their receptors locally within target tissues; and 6) to directly modulate cellular function, independent of their ability to bind IGFs. All six IGFBPs have been shown to inhibit IGF action, likely by limiting bioavailable free IGFs from interacting with their receptors. IGFBP-1 and IGFBP-3 can also be stimulatory to IGF action, presumably by forming a pool of “slow-release” IGFs. IGFBP-1 and IGFBP-3 additionally have IGF-independent actions, including alteration of cellular motility and inhibition of DNA synthesis, respectively. IGFBP-4 and -5 may also have IGF-independent actions both in the human ovary (431) and in cell lines derived from other tissues (430, 432). Since the affinities of IGFBPs 1–6 for the IGFs are equal to or greater than the affinities of the type I and type II IGF receptors for the peptides, mechanisms have evolved to decrease IGFBP affinities and increase IGF bioavailability to the receptors. These mechanisms include phosphorylation, glycosylation, and proteolysis (329).

This review will focus on IGFBP expression and regulation primarily in the human and rat ovary and underscore the mechanisms of ovarian IGFBP production and regulation common to other species. Also discussed are IGFBP proteolysis by specific proteases, the regulation of these enzymes,

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and their putative functions in normal and pathological ovarian conditions.

B. IGFBP expression in the ovary

IGFBPs are expressed by granulosa and thecal cells and are present in the FF of every species studied. Significant differences exist in the patterns of ovarian expression and regulation of individual IGFBP species between the human and animal models.

1. Human (Table 2). The human ovary expresses mRNAs for IGFBP-1, -2, -3, -4, and -5. *In situ* hybridization shows distinctive patterns of mRNA expression for each of these IGFBPs in antral follicles, with parallel localization of immunostainable protein (89). IGFBP-1 is localized only to the granulosa cells of dominant follicles, not to theca or small antral follicles. IGFBP-2 is expressed by granulosa cells only in small, nondominant antral follicles, but by thecal cells in both dominant and nondominant follicles. IGFBP-3 expression is found in the theca of all follicles and the granulosa of only dominant follicles. IGFBP-4 is found in both granulosa and theca in all follicles, with a slight increase in granulosa expression in dominant compared with small follicles. IGFBP-5 has also been localized to both granulosa and theca; its expression is unaffected by follicular development. No IGFBP-6 mRNA or protein was localized by *in situ* hybridization (89), but expression was detected by RT-PCR (347). A recent study found IGFBP-4 to be expressed in luteal cells and in the granulosa and theca layers of only atretic antral, not healthy or preantral follicles (433). The expression of IGFBP-2, -4, and -5 by both granulosa and thecal cells has been confirmed by Northern analysis (347). Expression of IGFBP-1 has also been found in the corpus luteum (434).

The regulation of IGFBP production by the human ovary has been examined in cell culture studies. Two sources of tissue have been employed: antral follicles from surgically excised ovaries, and granulosa-luteal cells obtained at oocyte harvest for *in vitro* fertilization (IVF) after controlled ovarian hyperstimulation (COH). Granulosa cells derived from antral follicles in spontaneous cycles release IGFBP-2 and both core and glycosylated isoforms of IGFBP-4 and express the corresponding mRNAs (347, 435, 436). Cultures of thecal tissue derived from these follicles produce IGFBP-2, -3, and -4; theca from mature healthy follicles also produces proteolytic fragments of IGFBP-3 and -4 (436–438). Thecal IGFBP-3 accumulation, as determined by ligand blotting, was stimulated markedly by LH/hCG or GH in one study (438), but these effects were not noted by others (347, 437). Thecal expression of mRNA for IGFBP-5, but not IGFBP-1, -2, -3, or -4, is stimulated by LH (347).

Because luteinizing granulosa cells from IVF oocyte harvests are readily available, this model has been extensively employed to study human IGFBP production. These cells express mRNAs for IGFBP-1, -2, -3, -4, and -5 in culture and accumulate all of these proteins except IGFBP-5, as detected by ligand blotting of conditioned medium (403, 439–443). By metabolic labeling, they synthesize IGFBP-1 and -2 *de novo*, but evidence for IGFBP-3 synthesis is conflicting (403, 444, 445). Although IGFBP-5 mRNA is abundantly expressed (442), no immunoprecipitable IGFBP-5 protein has been detected in conditioned medium (443, 446). These findings suggest that human granulosa cells elaborate an IGFBP-5 protease as has been reported in the rat (447, 448).

Production of each IGFBP species by human luteinizing granulosa cells is uniquely regulated. IGFBP-1 production is inhibited by FSH, insulin, IGF-I, IGF-II, and the somatostatin analog octreotide, and increased by LH, EGF, PGs, and phorbol ester (199, 396, 439, 449–454). The inhibition by insulin is mediated through its cognate receptor, not the type I IGF receptor (199). Both IGF-I and IGF-II inhibit IGFBP-1 production more potently than insulin (199, 449, 455) and apparently act via the type I IGF receptor. In fact, the concentrations of IGFs present in human FF completely inhibit *in vitro* granulosa cell IGFBP-1 production. This finding may explain the production of IGFBP-1 in cultured, but not in freshly obtained, human granulosa cells (347), as well as the observation that IGFBP-1 mRNA is not expressed in granulosa cells of small antral follicles (89). IGFBP-2 production is negatively regulated by LH/hCG through increased cAMP; this effect can be reversed by activin-A or interferon-γ (IFN-γ) (403, 443). IGF-II, but not IGF-I, decreases medium IGFBP-2, possibly through an action at the type II IGF receptor (403). In two studies, cAMP agonists promoted the accumulation of IGFBP-3 (403, 456), while a third found that FSH did not alter accumulation of immunoreactive IGFBP-3 but decreased its level on ligand blots, consistent with the action of an IGFBP-3 protease (451). In another study, IGFBP-3 detected by ligand blotting accumulated in conditioned medium during treatment with IGF peptides but not insulin, possibly reflecting release of IGFBP-3 from the cell surface upon binding ligand or protection from proteolysis (403). IGFBP-4 accumulation is inhibited by LH despite modest stimulation of its mRNA, apparently through elaboration of an IGFBP-4 protease (see *Section IV.C* below) (435, 436, 443, 457). IGFBP-5 mRNA expression is stimulated by activin-A (442).

IGFBPs found in human FF may either originate from local production or may reach the FF from an extraovarian source, such as the liver. FF IGFBPs have been measured both in antral follicles from cycling women and in hyperstimulated follicles aspirated for IVF, using both immunoassay and ligand blot techniques. FF from cycling women contains immunoassayable IGFBP-1, -2, and -3. IGFBP-1 levels range from 5–32 ng/ml, with levels positively correlated with follicular size and greater in dominant than cohort follicles (348, 446, 458). In one report, FF contained 15 ng/ml IGFBP-2, but the type of follicle studied was not stated (446). Mean immunoassayable IGFBP-3 in estrogen-dominant follicles (2995 ng/ml) was greater than in androgen-dominant follicles (2352 ng/ml); these levels were indistinguishable from those in hyperstimulated follicles (348). Immunoassays for IGFBP-4, -5, and -6 in these follicles have not been reported.

By ligand blotting, two distinct IGFBP profiles have been consistently observed in FF from cycling women (446, 459, 460). FF from estrogen-dominant, presumably healthy follicles contains low levels, while FF from androgen-dominant, presumably atretic follicles contains significantly greater levels of IGFBP-2 and both isoforms of IGFBP-4. The lower level of IGFBP-4 detectable by ligand blotting in FF from estro-

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genic compared with androgenic follicles results from the action of a serine metalloprotease found in estrogenic but not androgenic FF (see below) (435, 436, 457). An IGFBP-2 protease was also recently reported in estrogenic FF (436), but negative regulation of IGFBP-2 gene expression by gonadotropins (443) probably plays a more significant role in reducing IGFBP-2 levels in the healthy follicle. By contrast, IGFBP-3 levels are similar in FF from both types of follicles. In one study, IGFBP-3 levels in dominant follicles declined slightly but significantly with advancing follicle size and cycle day (446). IGFBP-1 has not been detected on ligand blots of FF from spontaneously cycling women (459).

FF obtained after hyperstimulation with menopausal gonadotropins followed by hCG contains IGFBP-1, -2, and -3, identified by immunoprecipitation (352, 434, 461). By immunoassay, mean IGFBP-1 levels are 90–160 ng/ml (434, 456, 462, 463), while mean IGFBP-3 levels are consistently near 2400 ng/ml (462, 464, 465), and IGFBP-6 levels are 170 ng/ml (466). By ligand blotting, IGFBP-1, -2, and -3 are detectable in FF from hyperstimulated cycles (352, 467).

2. Rodent. IGFBPs 2–6 have been detected in the rat ovary in both localization and cell culture studies (468–470). Studies of the cycling ovary revealed that IGFBP-4 and -5 are the predominant species expressed in granulosa cells of antral follicles. Both are preferentially localized to atretic follicles, with IGFBP-4 mRNA signal intensity increasing with the degree of atresia, and both IGFBP-4 and IGFBP-5 mRNA expression becoming more widespread in atretic follicles after the proestrous gonadotropin surge (468–470). In PMSG/hCG-treated rats, each gonadotropin treatment increased IGFBP-4 mRNA expression in small antral follicles, but no expression was seen in large follicles (471). Cultured granulosa cells from immature, diethylstilbestrol (DES)-treated rats secrete intact IGFBP-4 and IGFBP-5 into the medium (447, 448, 472). These cells respond to saturating doses of FSH by decreasing accumulation of both IGFBP-4 and IGFBP-5. These effects result from both decreases in mRNA expression and increases in elaboration of protease activities that degrade these IGFBPs into smaller, inactive fragments (448, 460, 473). Paradoxically, low doses of FSH (1–3 ng/ml) stimulate IGFBP-4 and -5 release (460). GnRH agonists, which induce follicular atresia (473) and granulosa cell apoptosis (474), stimulate basal IGFBP-4 accumulation without affecting IGFBP-4 protease activity and block the effect of FSH on both IGFBP-4 production and protease activity (473). IGF-I stimulates IGFBP-5 accumulation and decreases IGFBP-5 protease elaboration, while GnRH agonists can oppose the effects of FSH on both IGFBP-5 mRNA and protein expression and IGFBP-5 protease elaboration (447, 475, 476). Cytokines and growth factors known to block FSH-induced estradiol production, including TGF-β, tumor necrosis factor (TNF)-α, basic fibroblast growth factor, and interleukin-1α, stimulate IGFBP-4 (477), suggesting that their effects on FSH action are due to the IGF-I-sequestering properties of IGFBP-4. Activin-A can decrease both IGFBP-4 and IGFBP-5 mRNA expression and IGFBP-5 protein accumulation (478).

In contrast to the expression of IGFBP-4 and -5 by granulosa cells, IGFBP-2 mRNA expression and production in culture are unique to theca-interstitial cells in the rat ovary. IGFBP-3 expression is limited to theca-interstitial cells and vascular and perivascular elements of corpora lutea, suggesting that it plays a role in the vascular control of luteal regression (468, 479–481). IGFBP-6 expression is limited to the thecal layer (422), while no IGFBP-1 expression has been detected (448, 468).

IGFBP production has also been examined in the mouse ovary. Notable differences from the rat include expression of IGFBP-2 by granulosa cells (364, 367), negative correlation of granulosa IGFBP-5 expression in antral follicles with atresia (367), and the failure of FSH to inhibit accumulation of IGFBP-4 and -5 in granulosa cell-conditioned medium (364, 367). In the mouse ovary, expression of IGFBP-4 was increased in granulosa cells of histologically atretic follicles and was correlated with positive staining for the DNA fragmentation characteristic of apoptosis (367).

3. Livestock species. The pig ovary expresses IGFBP-2, -3, -4, and -5, with granulosa cell IGFBP-2 localized by *in situ* hybridization to small follicles and IGFBP-4 to large follicles (482). IGFBP-2 mRNA and protein levels decline with advancing follicular development (483). Cultured porcine granulosa cells elaborate both IGFBP-2 and -3, with production of IGFBP-3 and IGFBP-2 stimulated by IGF-I and decreased by FSH (484, 485). Granulosa cells from medium-sized follicles also accumulate IGFBP-4 and -5. IGF-I stimulates, while FSH inhibits, IGFBP-5 mRNA and protein production. FSH stimulates elaboration of 22-kDa IGFBP-4 (484, 486). In porcine FF, follicular growth is accompanied by a slight increase in IGFBP-3 and a decrease in IGFBP-2 and IGFBP-4, as assessed by ligand blotting (487–489). While IGFBP-4 and IGFBP-5 are undetectable in FF from preovulatory follicles, atresia is associated with a marked increase of intrafollicular levels of IGFBP-2 and IGFBP-4 (487, 489, 490).

In the sheep, IGFBP-4 and -5 expression in healthy follicles is mainly limited to the theca (491–493). In atretic follicles, both IGFBP-2 and -5 are more strongly expressed in the granulosa layer than in healthy follicles, while both IGFBP-2 and -4 are more strongly expressed by the theca (493). FF content of IGFBP-2 and -4 declines, while IGFBP-3 slightly increases, with follicle growth. Atresia is associated with increased content of IGFBP-2, -4, and -5 (424, 493).

In the cow, as in the sheep, IGFBP-2, -3, -4, and -5 have been identified in FF by immunoprecipitation. By ligand blotting and mRNA expression analysis, IGFBP-2 and -4 are more abundant in estrogen-poor, atretic follicles than in estrogen-rich, healthy ones (384, 387, 494–497). Within the dominant follicle, an increase in IGF-I and IGF-II with a concomitant decrease in IGFBP-2 may promote follicular dominance (388).

In summary, since granulosa cells from the pig, sheep, and cow express IGFBP-2, these three livestock species are better models for the human ovary than is the rat. The large animal models also permit the study of FF IGFBP content in relation to follicular functional status. In every species in which such studies have been reported, atretic follicles contain higher levels of IGFBPs -2, -4, and/or -5. Additionally, in cell culture models, gonadotropins universally decrease accumulation by granulosa cells of these small IGFBPs. These findings suggest that in a highly conserved mechanism, IGFBPs -2, -4,

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and -5 serve as IGF antagonists in follicles destined to undergo atresia, and that gonadotropins may exert their antiatretic action in part through down-regulation of IGFBP production. By contrast, IGFBP-3 may reach FF from thecal production or from the circulation; its level in FF is not affected by gonadotropins or atresia, but rather increases modestly with follicular maturation. By contrast to the smaller IGFBPs, IGFBP-3 appears not to function as an IGF antagonist within the follicle, possibly because it is saturated with ligand.

C. IGFBP proteases in the ovary (Table 2)

IGFBP protease activity was first demonstrated for IGFBP-3 in human pregnancy serum (498, 499). Subsequent reports of IGFBP-3 protease activity in pregnancy serum of other species (500, 501) were followed by nearly a decade of discovery of IGFBP proteases, which exist for most of the IGFBP species in a variety of biological fluids and are produced and secreted by a variety of cell types (329, 430, 502). The IGFBP proteases comprise a superfamily that includes several classes of proteases, including metal-dependent proteases, matrix metalloproteinases, disintegrin metalloproteinases, kallikreins, and cathepsins. These molecules likely represent enzymes with multiple active sites, multimeric proteins with subunit-specific active sites, or a cascade of enzymes with different activities. Several IGFBP proteases have been characterized with regard to their active sites and cofactor requirements, and the human pregnancy serum IGFBP-3 protease has been purified and characterized as a disintegrin metalloproteinase (503). Most IGFBP proteases are specific for particular binding-protein substrates. IGFBP-3 is the most susceptible to proteolysis by a variety of proteases, whereas IGFBP-1 appears to be the most resistant (504). Sequence analyses of IGFBP cleavage sites suggests that most proteolysis occurs in nonconserved regions (505).

The proteolysis of IGFBPs is likely to be an essential mechanism in the complex regulation of IGF action. IGFBP proteases partially proteolyze IGFBPs, resulting in lowered affinities of the IGFBP fragments for IGF peptides, thus increasing IGF binding to their receptors. In support of this concept, inhibitory effects of IGFBPs on IGF-stimulated DNA synthesis and mitogenesis are reversed in the presence of IGFBP protease activity in cultured chick embryo fibroblasts and prostatic epithelial cells, respectively (506, 507). In serum, proteolysis of IGFBP-3 releases IGFs for transport to the extravascular space, where they are likely bound to other IGFBPs, which are subsequently cleaved to promote release of the IGFs for action within the tissue. IGFBP-3 fragments may act at the cell membrane to augment the stimulatory effects of IGFs (508). Spatial and temporal regulation of IGFBP proteases is essential for controlled IGF actions, as well as the actions of IGFBP fragments.

It is remarkable that IGFBP-4 protease activity has been found in the ovaries of all species examined, including the pig, cow, and sheep. In these livestock species, the patterns of expression of low mol wt IGFBPs and their proteases in atretic and growing follicles are similar to those observed in follicles of other species. Likely this finding reflects a conserved mechanism that has evolved to regulate IGF bioavailability in the ovarian follicle (509–511). In the next sections, we will review the IGFBP protease activities that have implications for ovarian function in human and rat ovaries.

1. Human.

a. IGFBP-4 protease. IGFBP-4 exists as a nonglycosylated 25-kDa form and a 32- to 34-kDa glycosylated protein. While some IGFBPs have inhibitory as well as stimulatory effects on IGF actions, IGFBP-4 appears to have exclusively inhibitory actions (429). IGFBP-4 mRNA and protein are abundantly expressed in small antral (androgen-dominant) follicles of normal and polycystic human ovaries (89, 343). As noted above, the apparent absence by ligand blotting of IGFBP-4 in FF from estrogen-dominant, compared with androgen-dominant, follicles (446, 459, 460, 512) was demonstrated to be due to an IGFBP-4 protease that decreases the affinity of IGFBP-4 for IGFs (457, 513). This protease is a metal-dependent enzyme with a pH optimum between 7 and 9 (436, 457), which is produced by nonluteinizing granulosa cells before the LH surge as well as by luteinizing granulosa (436, 443, 457, 513). The degree of proteolysis of IGFBP-4 is inversely proportional to the A:E ratio within the follicle (513). IGFBP-4 protease activity is stimulated by gonadotropins, IGF-I and -II, activin-A, and IFN-γ (435, 443, 513); FSH and IGF-II synergistically stimulate this activity in nonluteinizing granulosa cells (435).

When unsaturated with IGF peptide, IGFBP-3 inhibits proteolysis of IGFBP-4, whereas when saturated, it permits IGFBP-4 proteolysis (514). The implication of this finding is that in estrogen-dominant follicles, where IGF levels are high and IGFBP-3 is presumably saturated, IGFBP-4 proteolysis can increase IGF bioavailability from the pool of IGFs bound to this binding protein. In contrast, in androgen-dominant follicles, where IGFBP-3 is presumably unsaturated due to low levels of IGF production, any IGFBP-4 protease activity present is inhibited by the unsaturated IGFBP-3.

b. IGFBP-3 and IGFBP-2 proteases. IGFBP-3 protease in estrogen-dominant FF (FFe) obtained at oocyte harvest from patients undergoing IVF was first demonstrated by Gargosky *et al.* (465). Iwashita *et al.* (515) also demonstrated a protease in FFe that cleaved radiolabeled IGFBP-3 into smaller fragments, whose activity in medium conditioned by luteinizing granulosa cells was stimulated by increasing doses of FSH. A 29-kDa fragment of IGFBP-3 was found in FF from dominant, compared with small antral, follicles, consistent with the presence of an IGFBP-3 protease (436, 465). With regard to IGFBP-2, immunoblotting revealed almost exclusively a 23-kDa IGFBP-2 fragment in FF from dominant follicles, compared with nearly exclusively intact IGFBP-2 and minimal fragments in FF from small cohort follicles (436). These observations are consistent with an IGFBP-2 protease in FFe, although specific IGFBP-2 proteolysis has not yet been demonstrated in these follicles. FSH action on luteinizing granulosa cells increases IGFBP-3 immunoreactivity in conditioned medium and apparently also increases IGFBP-3 proteolysis. These effects were found to be dose-dependent (515). These observations underscore the

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complexity of the mechanisms underlying control of IGF bioavailability within the human follicle.

c. Thecal and stromal proteases. Limited information is available regarding IGFBP protease in the thecal or stromal compartments of the ovary of humans or other species. In human thecal cell-conditioned medium, LH decreases IGFBP-2, -3, and -4 levels, but no increase in low molecular weight forms consistent with proteolysis was seen. Conditioned medium contains an IGFBP-3 protease, which was partially inhibited by metal chelators. No difference was observed in theca from patients with normal or polycystic ovaries (438, 516).

In summary, since IGFs are potent stimulators of steroidogenesis and follicular growth in the human ovary, their regulation by IGFBPs and IGFBP proteases is temporally and spatially related within ovarian tissues. This is likely to provide timed promotion and inhibition of growth factor action during periods of follicular development and of limited follicular growth or steroidogenesis, respectively.

2. Rodent. Cultured rat granulosa cells secrete intact IGFBP-4 and IGFBP-5 into the medium (see above). When rat granulosa cells are cultured with FSH, there is a dose-dependent decrease in intact IGFBP-4 and an increase in a 17.5-kDa IGFBP-4 fragment, suggesting the stimulation of an IGFBP-4 protease by FSH (448, 473, 517). This proteolytic activity has a neutral pH optimum and is inhibited by EDTA, but not by other protease inhibitors, suggesting its dependence on a divalent cation (517). Some studies, however, failed to find IGFBP-4 protease activity in granulosa cell-conditioned medium, regardless of FSH stimulation (447, 518). FSH, but not IGF-I, also stimulates proteolysis of IGFBP-5. The granulosa-derived IGFBP-5 protease appears to be a zinc-dependent metalloprotease of molecular mass greater than 100 kDa, which is specific for IGFBP-5. The resulting degradation fragments were estimated at 18 and 14 kDa in one study (447) and 19.5 and 17.5 kDa in another (518). Under cell-free conditions, IGF-I attenuates IGFBP-5 proteolysis, suggesting that binding to IGF-I may be protective (447, 518). GnRH, which increases IGFBP-4 and IGFBP-5, does not induce protease activity for either of these IGFBPs under basal conditions, but it completely blocks the ability of FSH to inhibit IGFBP-4 and IGFBP-5 accumulation and stimulate protease activity (473, 476, 518). Since IGFBP-4 and IGFBP-5 are effective inhibitors of FSH action in rat granulosa cells, regulated production of their proteases is likely to be important in FSH-dependent control of follicle growth and development.

In summary, IGFBP proteases are produced by granulosa and theca cells at distinct times of follicle development in ovaries from a variety of species. This conservation of expression and their regulation by gonadotropins, IGFs, and other peptides and cytokines underscore the importance of IGFBP proteases in regulating IGF bioactivity at unique stages of follicle development. The striking absence of IGFBP-4 protease in androgen-dominant follicles and the presence of this enzymatic activity in estrogen-dominant follicles argue strongly for an important role for the IGF peptides as co-gonadotropins and for IGFBPs as antigonadotropins during follicular growth, steroidogenesis, and atresia.

D. IGFBP actions in the ovary

Studies of IGFBP actions in the ovary have largely employed IGFBPs purified from the FF of large animals or prepared by recombinant DNA technology, with cultured granulosa cells from DES-primed, immature rats as the target. When IGFBP-1, -2, -3, or -4 is added to cultured rat granulosa cells, each can inhibit FSH-stimulated steroidogenesis (471, 519, 520), while IGFBP-6 is ineffective (422). Porcine IGFBP-3 and IGFBP-2 inhibit FSH-stimulated E_2 and P release; their lack of efficacy in the presence of IGF-I antiserum or IGF peptide suggests that they act by neutralizing endogenous IGF-I (471, 519, 521). In this model, IGFBPs also decrease mitosis and cAMP generation. Human IGFBP-1, -2, -3, and -4 all similarly decrease FSH-stimulated P output (471, 522); human IGFBP-6 does not, possibly because of its lower affinity for IGF-I, the principal IGF produced by rat granulosa cells, compared with IGF-II (422). The physiological relevance of IGFBP actions on the granulosa is strongly suggested by *in vitro* studies showing the greater potency of IGF peptide analogs that do not bind to IGFBPs, compared with the native peptides, only under conditions of high-medium IGFBP levels (522). These observations have led to the conclusion that intrinsic IGF-I is an obligatory mediator of FSH-induced E_2 and P production by rat granulosa. Additional *in vivo* evidence for the biological relevance of IGFBP action on the ovary comes from studies showing that injection of IGFBP-3 into the rat ovarian bursa or introduction of IGFBP-3 into the *in vitro* perfusate of rabbit ovaries each can decrease the rate of follicular rupture at ovulation (523, 524), and from the recent observation that transgenic mice overexpressing IGFBP-1 have reduced numbers of ovulations per estrous cycle (525).

IGFBP actions on human granulosa cells are similar to those on cells from the rat. In cultured granulosa-luteal cells, IGFBP-1 and -3 decrease IGF-I-stimulated E_2 production; IGFBP-1 also decreases IGF-I-stimulated mitosis (390, 399, 513, 526, 527). IGFBP-3 fails to inhibit the steroidogenic effect of des(1–3)IGF-I, an analog that does not bind to IGFBPs. In granulosa cells obtained from women during unstimulated cycles, IGFBP-1 and IGFBP-3 inhibit IGF-I-stimulated E_2 and P production (399).

Recombinant human (rh) IGFBP-4 inhibits IGF-stimulated E_2 production by human granulosa cells (431, 435, 512, 513). Iwashita *et al*. (513) employed luteinizing granulosa cells, whereas Chandrasekher *et al*. (435) and Mason *et al.* (512) used nonluteinizing granulosa cells, showing that rhIGFBP-4 can inhibit both IGF-II- and FSH-stimulated E_2 production. This inhibition exceeded 80%, while in similar experiments IGFBP-2 or IGFBP-3 inhibited granulosa cell steroidogenesis by only about 20% (512). In contrast to the inhibitory effects of intact rhIGFBP-4 on E_2 production, addition of proteolyzed IGFBP-4 was without effect (513). These findings support an important role for IGFBP-4 and IGFBP-4 protease in the regulation of follicular steroidogenesis in the human ovary. IGFBP-4 inhibits FSH-stimulated E_2 production in the absence of added IGF peptide or in the presence of type I IGF receptor antibody, suggesting either IGF-independent action or antagonism of a locally produced IGF (431, 435, 512). Nevertheless,

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IGFBPs consistently display actions on cultured ovarian tissues opposite to those of IGF peptides and gonadotropins, suggesting that an excess of IGFBPs can be antigonadotropic (409, 528) and result in either follicular arrest (as in PCOS) or atresia.

In addition to regulating follicular differentiation and maturation, IGFs and IGFBPs also likely play a role in regulating apoptosis of granulosa cells, which is associated with follicular atresia (201). In a rat antral follicle culture system, both gonadotropins and IGF-I can prevent the apoptosis of granulosa cells that occurs spontaneously in serum-free medium, and IGFBP-3 reverses the protection from apoptosis afforded by hCG, FSH, GH, and IGF-I (200, 529). The restriction of IGFBP-4 expression in the mouse follicle to histochemically apoptotic granulosa cells (367) also supports a role for IGFBPs in promoting follicular atresia *in vivo*.

E. Role of IGFBPs in follicular development and atresia

In the growing estrogen-dominant follicle, a number of mechanisms have evolved to increase IGF peptide bioavailability and thereby amplify granulosa responsiveness to the growth-promoting, steroidogenesis-promoting, and anti-apoptotic actions of FSH (Fig. 6). These include up-regulation of IGF receptors by gonadotropins and, in the rat, by estrogens (90, 530, 531); increase in IGF expression by gonadotropins (345, 532); inhibition by IGFs and gonadotropins of inhibitory IGFBP synthesis (403); and stimulation by gonadotropins and IGF-II of IGFBP protease activity (435, 513). The net result is maximum bioavailability of IGF peptides. In contrast, in the androgen-dominant follicle that is arrested in development or destined for atresia, these mechanisms are reversed (Fig. 6): FSH receptor numbers are low; IGF expression is almost undetectable; there is abundant expression of inhibitory IGFBPs (IGFBP-2 and IGFBP-4); and there is minimal detectable IGFBP protease activity. The net result is that aromatase is not induced, and thus precursor androgen persists in these follicles, in association with developmental arrest or atresia.

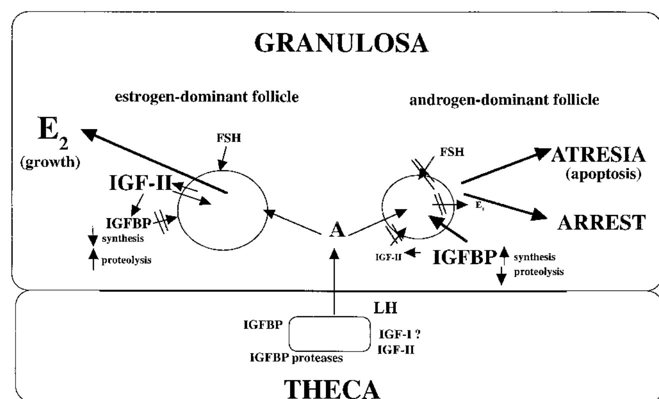

FIG. 6. Model of IGF, IGFBP, and IGFBP protease actions in human ovary. In the estrogen-dominant, healthy growing follicle (shown at *top left*), granulosa cell IGF-II production increases, synergizing with FSH. IGF-II action is amplified by decreased synthesis and increased proteolysis of IGFBPs. In the androgen-dominant follicle (shown at *top right*), both increased IGFBP synthesis and decreased IGFBP proteolysis contribute to decreased FSH and IGF-II action on the granulosa, resulting in atresia or developmental arrest.

The question remains, however, whether relative IGFBP expression is causally involved in selection and maturation of the dominant follicle. The study of IGFBPs in PCOS (see *Section V*) had been anticipated to shed some light on their role in follicular maturation in this disorder. Women with PCOS appear to have a defect in antral follicular maturation, but the cause of this defect has not been identified. Levels of IGFBPs in FF and IGFBP mRNA expression in follicular cells of the PCOS ovary are similar to those in small antral (largely atretic) follicles in normal women (89, 347, 533, 534). This appears to exclude a unique defect in IGFBP regulation in the ovary as a cause of the PCOS follicular maturation defect. Rather, in both the PCOS and normal ovary, the challenge is to explain how FSH can be successful in suppressing IGFBP production in one follicle (destined for dominance) while failing to do so in others (cohort follicles destined for atresia).

F. Summary

The high levels of expression of IGFs and low levels of expression of inhibitory IGFBPs in healthy follicles, and the reverse in atretic follicles, suggest that the level of bioavailable IGFs may play a role in regulating follicular growth, steroidogenesis, and apoptosis. IGFBPs and IGFBP proteases could thus assume importance in determining follicular destiny, since they can modulate the bioactivity of members of the IGF family.

V. Polycystic Ovary Syndrome (PCOS)

A. Clinical features

PCOS is a disorder of unknown, probably heterogeneous, etiology, characterized by chronic anovulation, biochemical and/or clinical evidence of hyperandrogenism, and enlarged, polycystic ovaries (535, 536). When first described by Stein and Leventhal (537) in 1935, the syndrome was defined by ovarian enlargement and multiple small cysts, in association with amenorrhea and hirsutism. PCOS affects between 5–10% of women of reproductive age (538, 539), and the onset of clinical manifestations often occurs at the time of puberty (191). In recent years, varying definitions of this syndrome have been used in studies of this disorder, with some investigators requiring polycystic ovaries on ultrasound for inclusion, and others requiring an elevation of serum LH or LH:FSH ratio (540). A consensus definition of PCOS was reached in 1990 under NIH auspices, which requires only hyperandrogenism of ovarian origin and oligomenorrhea or amenorrhea, with exclusion of other specific disorders such as steroid 21-hydroxylase deficiency (541). Other endocrine abnormalities that are inconsistently present in women with PCOS include obesity, peripheral insulin resistance and hyperinsulinemia, and elevations of serum PRL or DHEA-sulfate. Phenotypic differences among PCOS study populations may reflect underlying genetic differences in etiology or pathophysiology or in peripheral manifestations such as hirsutism (542, 543). Differences in diagnostic selection criteria can make comparison of studies on PCOS difficult.

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PCOS is perhaps the most common disorder in which the association between insulin resistance and ovarian function appears to be important. Since several comprehensive reviews on this subject are available (26, 27, 140, 535), we focus herein on the controversial issues related to the pathogenesis of PCOS and the changes in the insulin-related ovarian regulatory system observed in PCOS. In the following section, we will review recent studies that have evaluated the use of inhibitors of insulin secretion and insulin-sensitizing agents in the therapy of PCOS.

B. Theories of pathogenesis

Determining the etiology or etiologies of PCOS has proven elusive. It was recognized as early as 1980 by Yen (544) that in PCOS a number of endocrine abnormalities perpetuate themselves in what has been described as a "vicious cycle." These include abnormal gonadotropin secretion, with excess circulating LH and low, tonic FSH levels; hypersecretion by ovarian thecal and stromal compartments of androgens, which were viewed as both disrupting follicular maturation and providing substrate for peripheral aromatization to estrogens in adipose and other sites; and negative feedback of this tonic estrogen production on the pituitary to decrease FSH secretion and thus trophic support of the granulosa cell (544). The vicious cycle concept was further supported by studies suggesting that normal ovulatory function can occur after disruption of this cycle, *e.g.*, by ovarian wedge resection or cautery or during recovery from GnRHa-induced suppression (545–548). The vicious cycle concept does not, however, provide an explanation of how the abnormalities become established. A number of endocrine disorders can produce similar anovulatory, hyperandrogenic states, such as functional or drug-induced hyperprolactinemia (549, 550) and adult-onset congenital adrenal hyperplasia resulting from 21-hydroxylase deficiency (551, 552). The primary abnormality in PCOS has been proposed to be of central, ovarian, adrenal, or peripheral metabolic origin. These theories will be briefly reviewed below.

1. Central hypothesis. Abnormalities in LH-secretory pattern and its regulation have been observed in PCOS. Women with PCOS often have both increased LH pulse amplitude and frequency, compared with ovulatory controls (168, 553–555). This results in increased or disordered LH secretion and may lead to an elevated serum LH:FSH ratio. These central alterations may be mediated by the altered steroid milieu of PCOS rather than being primary, since during recovery from GnRHa suppression no difference was seen between PCOS and normal women in the recovery of LH pulse frequency (556). On the other hand, while P normally slows GnRH pulse frequency, women with PCOS appear relatively resistant to this effect (557, 558), and chronobiological abnormalities of LH secretion can be observed in adolescent girls with features of PCOS (559), suggesting a primary abnormality of GnRH pulsatility in this disorder.

Abnormally rapid GnRH pulse generation is assumed to underlie abnormal LH secretion in PCOS. Abnormalities in other neuroendocrine modulators, such as the endogenous opioids, dopamine and leptin, have also been proposed as determinants of gonadotropin secretion in PCOS. Endogenous opioid excess may sensitize the gonadotrope to GnRH, particularly in association with hyperinsulinemia (37, 560). Decreased dopaminergic inhibition of LH release (561) and an increased incidence of an allelic form of the D3 dopamine receptor have been noted in women with PCOS (562). Recently, the possible role of leptin in PCOS has been examined. An initial report found serum leptin levels in a small subpopulation of women with PCOS greater than predicted from their BMI (563), but subsequent reports have failed to confirm this finding (564–568). In one study, hyperinsulinemia was associated with increased circulating leptin in PCOS subjects (141), although no association of serum leptin and insulin in women with PCOS was found in two other studies (567, 569). It seems unlikely that leptin is responsible for increased LH secretion in PCOS, since either an inverse (565, 570) or no relationship (563, 566) has been reported between serum leptin and LH levels. At this time, it is unclear whether leptin plays a role in the etiology of PCOS.

2. Ovarian hypothesis. An intrinsic ovarian functional defect has also been postulated as the source of the self-sustaining abnormalities in PCOS. Thecal hypertrophy and overproduction of androgens are recognized features of the PCOS ovary. When placed in culture, PCOS thecal cells continue to hypersecrete androgens, and when deprived of trophic support through GnRHa suppression, the PCOS ovary continues to hypersecrete 17-OHP in response to hCG *in vivo* (571–573). Dynamic short-term GnRHa testing in PCOS produces an exaggerated ovarian 17-OHP-secretory response (107, 573, 574). This response may reflect the increased thecal mass present in the ovary, but has been also interpreted as reflecting dysregulation of the activity of the steroidogenic enzyme P_{450}c17, which is responsible for both 17-hydroxylation of C21 steroids and for the 17,20-lyase activity necessary for androgen (C19) synthesis (575). The recent report that the lyase activity of P_{450}c17 can be promoted by serine phosphorylation of the enzyme (576) suggests a possible mechanism for abnormal steroidogenesis in PCOS. It is intriguing that excessive serine phosphorylation of the insulin receptor has been proposed as a cause of peripheral insulin resistance in some women with PCOS (577) (see below; *Section V.C*).

Granulosa cell steroidogenic and mitogenic abnormalities have also been found in PCOS. Aromatase activity is low in PCOS granulosa cells *in vivo*, reflecting decreased FSH activity, but is normal or exaggerated when they are cultured (105, 578). This observation led to the concept that the PCOS follicle contains excessive amounts of inhibitor(s) of FSH action. While IGFBP-2 and -4 are FSH antagonists (471, 521) that are abundant in FF from PCOS antral follicles, their expression in the PCOS ovary is indistinguishable from that in the cycling ovary (89, 533, 579) (see above), weakening the argument for an etiological role of these proteins. Other studies suggested that an inhibin α-subunit-processing product, pro-αC, can serve as an FSH antagonist and is found in FF (580, 581), but its presence and role in PCOS follicles are unknown. Granulosa cell mitosis also appears defective, in that granulosa cell numbers in PCOS follicles are lower than in healthy size-matched follicles from cycling women (582),

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but whether abnormal granulosa cell mitosis is important in the pathogenesis of PCOS has not been directly tested.

3. Adrenal hypothesis. Many women with PCOS develop irregular menses shortly after menarche. It has been hypothesized that excessive production of adrenal androgens, which increases at puberty, can supply substrate for extragonadal aromatization and result in tonic estrogen inhibition of FSH secretion (544). Premature adrenarche is associated with a higher incidence of both functional ovarian hyperandrogenism, with exaggerated 17-OHP response to GnRHa challenge (583, 584), and insulin resistance (585, 586). Hyperinsulinemia can stimulate adrenal as well as ovarian steroidogenesis (587). Since insulin resistance accompanies puberty and may contribute to adrenarche, an important unanswered question is why pubertal insulin resistance fails to resolve in adolescent girls who develop PCOS, and whether the effect of hyperinsulinemia on the adrenal, on the ovary, or on both of these organs is significant in the pathogenesis of PCOS.

C. Insulin resistance in PCOS

1. Putative causes and role in pathogenesis. A majority of women with PCOS demonstrate peripheral insulin resistance involving skeletal muscle and adipose tissue, which results in compensatory hyperinsulinemia (140). Insulin resistance does not appear to involve ovarian steroidogenesis, because granulosa and thecal cells from PCOS ovaries demonstrate a normal dose response to insulin in culture (96, 97). As a result, excessive insulin stimulation may promote thecal androgen hypersecretion.

Insulin resistance can be determined by measuring insulin levels during frequently sampled IVGTT (588) or by euglycemic, hyperinsulinemic clamp studies (589). Obese women with PCOS are more insulin resistant than weight-matched controls (589–591), suggesting that obesity and PCOS exert independent effects on insulin resistance. Many studies have found insulin resistance in lean as well as obese subjects with PCOS (120, 168, 589, 592), although at least one study failed to confirm this finding (593), and normal insulin sensitivity can be restored in some obese women with PCOS with weight loss (155).

The molecular basis of insulin resistance in PCOS is a subject of active research and has recently been reviewed (140, 594). Pedigree studies have suggested a genetic basis of PCOS in some kindreds, with premature balding as the male phenotype (595). In these families, linkage to the variable number of tandem repeats locus upstream of the insulin gene has recently been demonstrated (596). Mutations in the insulin receptor gene or defects in its intrinsic tyrosine kinase activity are rarely found, and insulin receptor binding is normal (216, 597–605). The defect of insulin action in PCOS appears to be at the postbinding level and to involve glucose transport (603); it may be observed only in some cell types (*e.g.,* in adipocytes but not skin fibroblasts) (606) and may be accompanied by a defect in insulin-induced inhibition of lipolysis (605, 607). Several molecular mechanisms for the glucose transport defect have been suggested by recent studies. In one of these, abdominal adipocytes of PCOS subjects had a lower content of the GLUT4 glucose transporter than controls (608). Another noted that the insulin receptor in about half of women with PCOS is excessively phosphorylated on serine, a state that reduces signal transduction (577). In another report, PCOS adipocyte insulin sensitivity could be restored by an adenosine receptor agonist, suggesting that depletion of cellular adenosine may lead to insulin resistance (609). The correction of insulin resistance by a thiazolidinedione, troglitazone (35, 36), suggests that women with PCOS may be deficient in signal transduction through peroxisome proliferator-activated receptor-γ (PPAR-γ), the natural ligand for which appears to be a PG of the J series or an essential fatty acid (610, 611) (see below).

The potential links between hyperinsulinemia and the increased androgen production observed in PCOS (27, 612) have been discussed previously (*Section II.C*); they include direct stimulation of ovarian androgen secretion by insulin, possibly through stimulatory effects on the 17α-hydroxylase/17,20-lyase and P_{450}scc enzymes; direct stimulation of LH secretion by insulin or sensitization of LH-secreting pituitary cells to GnRH stimulation; up-regulation of ovarian type I IGF receptors with the amplification of IGF-I, IGF-II, and insulin actions in the ovary; decreased levels of SHBG, with concomitant elevation of free androgens; decreased IGFBP-1 production, both in the liver and in the ovary, with concomitant elevation of free IGFs in the circulation and in the ovary; and the synergistic growth- and cyst-promoting action of insulin and LH.

In addition to these effects, an action of insulin on granulosa cells has been implicated in the follicular developmental disorder of PCOS. Granulosa cell numbers are decreased relative to follicle size in PCOS (582), and it has recently been suggested that acquisition of granulosa cell LH responsiveness too early in follicular development may have an antiproliferative effect on granulosa cells in PCOS (613, 614). Hyperinsulinemia could accelerate development of granulosa cell LH responsiveness by amplifying the induction of LH receptors (13, 96, 164, 613).

It has been proposed both that hyperandrogenemia may contribute to insulin resistance in PCOS and that hyperinsulinemia can promote hyperandrogenism (3, 9, 120). The results of pharmacological modification studies have suggested that the latter mechanism is more operative than the former. Androgen levels in PCOS have been reduced and their action blocked by the use of GnRHa and androgen receptor blockers. Suppression of ovarian or adrenal steroidogenesis has not improved insulin resistance (615–617), although in some studies, antiandrogens such as flutamide and spironolactone (618–620) have led to partial improvement. Ovarian cautery, which lowers androgen secretion, does not alter insulin resistance (621). Direct administration of androgens to oophorectomized women has no effect on insulin levels, though it increases circulating levels of IGF-I and suppresses SHBG (622). On the other hand, pharmacological reduction in the level of hyperinsulinemia, either by insulin sensitizers such as metformin or troglitazone or by insulin secretion inhibitors such as octreotide or diazoxide, has consistently improved circulating androgen levels (29–31, 34–36, 108, 142, 143, 221, 623, 624). Additionally, the occurrence of hyperandrogenism in states of extreme insulin

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resistance other than PCOS (9, 140) and in association with hyperinsulinemia induced by valproate therapy for epilepsy (625) supports a primary role for insulin excess in producing ovarian dysfunction.

In addition to decreased insulin sensitivity, insulin secretion in patients with PCOS also appears to be abnormal (137, 626). In particular, early insulin release after ingestion of glucose appears to be exaggerated (155, 627, 628). A decrease in the amplitude of meal-related insulin pulses and defective insulin clearance in peripheral tissues have also been reported (629, 630). Patients with PCOS exhibit abnormal entrainment of insulin secretory pulses in response to an oscillatory glucose infusion (626). These abnormalities, however, may be secondary to insulin resistance, since they can be reversed with the use of insulin-sensitizing agents (36). Both obese and nonobese women with PCOS appear to have inadequate insulin secretion for their degree of insulin resistance (631), placing them at an increased risk for the development of type 2 diabetes (538).

2. Role of obesity in PCOS. Some aspects of insulin action in obesity resemble those seen in PCOS (632–635). Many patients with obesity are insulin resistant and hyperinsulinemic (636–638) and, when central obesity is present, often have reduced circulating levels of SHBG and mildly elevated androgen levels (639–644). Because insulin resistance has not been consistently encountered in populations of lean women with PCOS, the existence of a cause of insulin resistance in PCOS distinct from that associated with obesity remains open to question. That obesity contributes significantly to both insulin resistance and hyperandrogenism in overweight women with and without PCOS is evident from the improvement in androgen levels usually seen with weight loss, sometimes to levels observed in weight-matched ovulatory women (121, 147–155, 632, 633, 639, 645–647). Anovulatory hyperandrogenemic adolescents and adults are more insulin resistant than weight-matched ovulatory controls (191, 586, 635, 648–651). Since there is some evidence that androgens may contribute to insulin resistance, however (619, 620, 652–654), this finding fails to resolve the question of whether insulin resistance in PCOS is independent of obesity.

The cause of obesity-related insulin resistance is itself not well understood. As discussed above, obese individuals are usually insulin resistant, and in some individuals obesity may be a necessary factor for the development of diabetes. For example, Sigal *et al.* (655) recently demonstrated that glycine-arginine polymorphism in codon 972 of the IRS-I gene clusters with diabetes and obesity, suggesting that this polymorphism may predispose to the development of type 2 diabetes only if obesity is also present. A Pro115Gln activating mutation in the PPAR-γ2 receptor has been associated with obesity (656); activation of this receptor may reduce insulin resistance, and individuals with this mutation appear to have lower circulating insulin levels than obese individuals without this mutation. Recent studies have implicated the cytokine TNF-α as a contributor to insulin resistance in obesity (241, 657–666). In Native American Pimas, in whom insulin resistance and obesity are highly prevalent, and in whom oligomenorrhea is common (212, 634), a mutation closely linked to TNF-α has been associated with insulin resistance (667). TNF-α is produced by adipose tissue and stimulates IRS phosphorylation on serine, which in turn appears to inhibit insulin receptor tyrosine kinase and PI-3 kinase activation (659, 662, 668–671). Interestingly, TNF-α may also interfere with the action of IGF-I, although this effect of TNF-α may involve not only the inhibition of type I IGF-receptor tyrosine kinase, but also stimulation of IGFBP production (672). TNF-α can also inhibit expression and signaling through PPAR-γ (673, 674), which serves as a major target for thiazolidinediones; it is controversial whether thiazolidinediones block TNF-α inhibition of PPAR-γ expression (675). TNF-α can also inhibit the synergism between insulin and FSH in stimulating steroidogenesis (676). Although all of these findings are of great interest, the ability of TNF-α to induce insulin resistance *in vitro* or *in vivo* has not been firmly established (677–679). Further, circulating as well as FF TNF-α concentrations in PCOS appear to be similar to those in normal women (680, 681). Leptin may also contribute to the insulin resistance of obesity via mechanisms similar to TNF-α, but *ob/ob* mice, which lack functional leptin, develop insulin resistance (287). Furthermore, in the Zucker fatty rat, which lacks a functional leptin receptor, IRS-1 and -2 are down-regulated in the liver, leading to a dramatic reduction in PI-3 kinase activity in spite of the leptin resistance (289).

In summary, the cause of insulin resistance in women with PCOS appears to be, at least in part, related to obesity, and insulin resistance is not present in all women with PCOS (682). Whether there is a component of insulin resistance in PCOS independent of the insulin resistance of obesity will be clarified once the specific molecular mechanisms of insulin resistance in both of these conditions are better understood (670, 683, 684). It has been proposed that the pathogenesis of PCOS is different in obese and nonobese women, with insulin resistance and hyperinsulinemia playing a central role in obese patients, and abnormalities of the GH-IGF-I axis being important in PCOS in lean women (168, 685, 686).

D. Alterations of IGFs and IGFBPs in PCOS

1. Ovarian IGF production. By *in situ* hybridization and immunohistochemistry, the patterns of IGF-I and IGF-II mRNA and protein expression in the antral follicles of the PCOS ovary were identical to those of the small antral, nondominant follicles of cycling women (89). In human thecal cell cultures from PCOS ovaries, no differences were noted in IGF-I or IGF-II production compared with cultures derived from control women (438). In FF, levels of IGF-I in PCOS are similar to or slightly greater than in FF from cycling women (687, 688). To our knowledge, basal levels of IGF-II in FF have not been reported in PCOS. However, after gonadotropin stimulation for IVF, intrafollicular IGF-II levels are lower in PCOS than in control women, and IGF-II expression by granulosa cells is lower as well (689).

2. Ovarian IGFBP production. IGFBP production has been examined in the PCOS ovary. In an *in situ* hybridization study, each IGFBP displayed a pattern of mRNA expression identical to that seen in the small antral follicles of cycling women (89). In a recent study, IGFBP-4 localization in PCOS antral follicles correlated with insulin sensitivity: insulin-

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resistant women had greater IGFBP-4 staining in theca than in granulosa, while the reverse was seen in non-insulin-resistant subjects (433). Two groups have examined IGFBP production by cultured cells derived from PCOS ovaries. San Roman and Magoffin (579) reported the presence of IGFBP-3 in media conditioned by both granulosa and theca cell cultures from three women with PCOS, with levels declining after gonadotropin stimulation. TGF-β increased IGFBP-3 production by granulosa cells and antagonized the effect of FSH. In a similar study, another group found no detectable IGFBPs by ligand blotting and no IGFBP-3 or IGFBP-2 by immunoblotting in granulosa cell-conditioned medium, while thecal cell-conditioned medium from PCOS ovaries showed the same IGFBP profile as that derived from cycling women (438).

The three groups that examined IGFBP profiles by ligand blotting in FF from cycling women (see *Section IV.B.1*) also examined FF from women with PCOS (460, 533, 579). They all found FF IGFBP profiles in PCOS similar to those in the androgen-dominant follicles of cycling women: levels of IGFBP-2 and IGFBP-4 are markedly elevated in PCOS follicles compared with estrogen-dominant follicles. By contrast, no differences in IGFBP-3 levels were noted in FF from PCOS follicles, androgen-dominant follicles, and estrogen-dominant follicles from cycling women (459, 579). These findings indicate that the bioavailability of IGFs within the PCOS follicle, as in all androgen-dominant follicles, is likely lowered by higher IGFBP levels. Two studies have noted that a spontaneous preovulatory follicle found in a woman with PCOS had an IGFBP profile similar to that of the preovulatory follicles of cycling women (460, 690), suggesting that the expression of IGFBP-2 and IGFBP-4 can be regulated normally in some women with PCOS. FF IGFBP-1 levels have been studied in PCOS by immunoassay. Levels in size-matched PCOS follicles were 48% of those from cycling women (458), possibly reflecting a greater inhibitory effect of insulin on IGFBP-1 production.

3. Serum IGFs. Perhaps as a consequence of decreased serum IGFBP-1 levels, serum free IGF-I levels are elevated in PCOS (348, 691). This latter finding suggests that IGF-I may be more available to the theca in PCOS than in normal women and may contribute to the increased androgen production by the PCOS theca cell (692). Serum free IGF-I levels do not correlate with IGFBP-1 levels, however, arguing against a causal relationship between decreased serum IGFBP-1 and increased IGF bioavailability at the follicular level. Serum total IGF-I and IGF-II levels are not different between PCOS and normal women (348, 691).

4. Serum IGFBPs. The role of circulating IGFBPs in modulating normal ovarian function is uncertain, in view of the lack of cycle-dependent changes in serum IGFBP-1 and IGFBP-3 (348, 693) and the lack of evidence from selective venous catheterization for a significant ovarian contribution to serum levels of these IGFBPs (694). Conversely, it is likely that FF IGFBP levels can be influenced by changes in serum levels, since FF contains transudated serum proteins. Because of the availability of immunoassays, IGFBP-3 and IGFBP-1 have been most extensively studied in PCOS.

No difference has been found by immunoassay in serum IGFBP-3 levels between PCOS and ovulatory controls (168, 348, 695, 696). Similar integrated 24-h IGFBP-3 levels were found in obese and lean women with PCOS, which also did not differ from obese or lean controls (168). One study examined the effect on serum IGFBP-3 of octreotide, which decreases insulin secretion in hyperinsulinemic women with PCOS (142). In women with PCOS unselected for insulin resistance, octreotide increased serum IGFBP-3 levels by 42%, while decreasing serum IGF-I by 63%. No change in IGFBP-3 and a smaller but significant decrease in IGF-I were observed in control women (696). The effect of octreotide on insulin secretion cannot explain this decrease in serum IGFBP-3, since insulin does not modulate circulating IGFBP-3 (697). The decrease in serum IGF-I also cannot explain the increase in IGFBP-3, since IGF-I does not appear to regulate serum IGFBP-3 (698). Rather, these findings suggest a central alteration in the GH/IGF-I axis in PCOS (168, 699).

Women with PCOS, particularly if obese, have lower serum IGFBP-1 levels than their normally cycling counterparts or anovulatory women without PCOS (348, 444, 592, 691, 700, 701). Fasting serum IGFBP-1 levels are negatively correlated with serum insulin levels in all human subjects, including those with PCOS (192, 194, 198, 701). In women with PCOS, IGFBP-1 levels decline during both OGTTs and IVGTTs in a fashion mirroring the insulin response (190, 192). Weight loss increases serum IGFBP-1 (702), while ovarian electrocautery, which improves ovulatory function, and GnRHa suppression of ovarian steroid production each has no effect on serum IGFBP-1 or insulin sensitivity (546, 621, 703). Thus, serum IGFBP-1 levels reflect both short-term fluctuations in insulin levels (183) and the degree of peripheral insulin resistance. It has been proposed that IGFBP-1 levels in women with PCOS may be useful clinically as a marker for insulin resistance (621).

E. Summary

Multiple abnormalities of the components of the insulin-related ovarian regulatory system are present in PCOS. It remains to be confirmed whether any of these abnormalities are primary in the pathogenesis of PCOS and whether they play an important role in the development of hyperandrogenism and anovulation in this disorder.

VI. The Insulin-Related Ovarian Regulatory System: Implications for Therapy

If abnormalities of the insulin-related ovarian regulatory system are of clinical importance in patients with altered ovarian function, one would expect the reversal of these abnormalities to lead to clinical improvement (32). There are several types of therapeutic interventions that may influence the ovarian insulin-related regulatory system: low calorie diets and weight reduction; insulin-sensitizing agents, including metformin, troglitazone, β_3-adrenergic receptor agonists, and vanadate; inhibitors of insulin secretion, such as octreotide and diazoxide; promoters of insulin clearance, such as the opioid antagonist naltrexone; IGF-I and IGF-II; and GH, which can act both through its own receptors and

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by affecting IGF-I production in the liver and IGFBP production in both the liver and the ovary.

A. Treatment of PCOS

1. Dietary modification. In numerous studies of women with PCOS, caloric restriction (even without weight loss) or weight-reducing diets have resulted in normalization of insulin sensitivity and gonadotropin and androgen metabolism (including P_{450}scc and 17α-hydroxylase activity); improvement of acanthosis nigricans, which is commonly observed in obese insulin-resistant women (704–706); and restoration of ovulation (147–152, 154–156, 646, 702, 707). As discussed above, mechanisms underlying such improvements may include a decline of insulin-stimulated gonadotropin secretion as well as a reduction of the direct stimulatory effect of insulin on the ovary and/or the adrenal and alleviation of insulin-induced inhibition of both SHBG and IGFBP-1. Additionally, the reduction of leptin levels observed during caloric restriction may lead to the deactivation of the hypothalamic-pituitary-ovarian axis. In practice, however, sustained long-term weight loss using dietary intervention can be accomplished only in a small number of obese individuals (636). Therefore, other therapeutic approaches are usually needed.

2. Agents that lower circulating insulin without affecting insulin sensitivity. Both diazoxide and octreotide can directly inhibit pancreatic insulin secretion; these agents also reduce androgen levels, and octreotide has been shown to restore ovulation (30, 34, 142, 221, 623). The long-term use of these agents in PCOS, however, is not desirable, since they may worsen glucose tolerance and further increase the risk of developing diabetes (224, 538, 708).

Opioid antagonists such as naltrexone can decrease the insulin response during OGTT and may do so largely by increasing the rate of insulin clearance in a subset of women with PCOS who may have defective insulin clearance (37, 223, 709, 710); they do not affect glucose utilization during a clamp study (223). Although naltrexone treatment has not been associated with lowering of LH or androgens (37, 711, 712), improvements in both spontaneous ovulation and responsiveness to clomiphene have been noted in association with the decline in circulating insulin (38).

3. Insulin-sensitizing agents. The biguanide metformin is an insulin sensitizer that can reduce hyperglycemia in type 2 diabetes. Its mechanisms of action involve suppression of hepatic glucose output and improvement in insulin sensitivity in peripheral tissues (713–717). Metformin has also been reported to increase insulin receptor tyrosine kinase activity in vascular smooth muscle (718). Metformin does not appear to have a direct effect on ovarian steroidogenesis (719) or on synthesis of IGFBP-1 (720).

The effects of metformin on circulating levels of insulin, androgens, and gonadotropins and on ovulatory function have been examined in PCOS. In a dose of 500 mg three times daily for 4–8 weeks, metformin improved insulin sensitivity and decreased hyperinsulinemia, with integrated insulin secretion during OGTT decreasing by 35–40% (31). Along with the reduction of circulating insulin, SHBG was increased and serum LH and androgens, as well as the exaggerated 17-OHP secretory response to GnRHa, were reduced (29, 31, 108, 143, 145, 624). These improvements occurred in both obese and lean subjects and were noted in placebo-controlled studies (29, 108). In one of these, reduction in serum free T and LH was accompanied by restoration of menstrual cyclicity in 21 of 22 subjects, associated in most with ovulatory P levels (143). A recent report also suggests that metformin can improve the ovulatory response to clomiphene in PCOS (721). Metformin appears to exert its inhibitory effect on androgens by reducing hyperinsulinemia, which in turn leads to decreases in pituitary LH secretion, thecal androgen secretion, and an increase in SHBG. Several studies, however, have not found an improvement in insulin sensitivity or androgen metabolism in PCOS with metformin (153, 722–724). In one of these, weight was deliberately maintained at a controlled level (723). In another study, obese, hirsute women were treated with a low calorie (1500 kcal/day) diet and in a randomized fashion with either placebo or 850 mg metformin/day. Diet led to a reduction in insulin levels, a rise of SHBG, and a fall in free androgen levels, but metformin had no additional effect (153).

Troglitazone, a thiazolidinedione, decreases peripheral insulin resistance and is useful in the treatment of type 2 diabetes (725–728). Thiazolidinediones are high-affinity ligands for PPAR-γ (729, 730), a member of the steroid nuclear receptor superfamily, and are believed to exert their effect on insulin sensitivity by activating this receptor. Activation of PPAR-γ in adipocytes promotes their differentiation and increases the expression of the fatty acid binding protein aP2 (731–733), as well as uncoupling proteins (734–736), which act in mitochondria to uncouple oxidation and phosphorylation. PPAR-γ activation is promoted by insulin (733). It is not known how thiazolidinediones mediate insulin sensitivity. It has been suggested that they may act in part by antagonizing TNF-α-induced insulin resistance (660, 673, 733) or by leading to a decreased production of leptin (737, 738). In subjects with impaired glucose tolerance or frank diabetes, troglitazone improves glycemic control and decreases circulating insulin concentration (725, 727, 739). In obese nondiabetic humans, troglitazone increases glucose disposal rate and improves insulin sensitivity (740). It has been proposed that troglitazone can delay or prevent the development of type 2 diabetes in insulin-resistant individuals, including women with a history of gestational diabetes (740, 741).

In two studies of obese women with PCOS, defined by hyperandrogenemia and oligomenorrhea or amenorrhea, troglitazone decreased circulating insulin levels and increased insulin sensitivity (35, 36). Notably, troglitazone also decreased serum free T and increased SHBG levels, the latter apparently a direct result of the decline in circulating insulin. In a study in which subjects were not selected for glucose intolerance (35), serum LH also declined. This study noted that 2 of 21 women (9%) ovulated spontaneously on troglitazone, based on serum P elevation. When subjects were selected for impaired glucose tolerance (36), serum total T declined and the 17-OHP response to leuprolide was also decreased, but LH levels were unchanged. The return of ovulation was not reported. In this group of subjects, characterized by abnormal pancreatic β-cell entrainment of in-

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sulin secretion to an oscillatory glucose infusion, troglitazone normalized the insulin-secretory response (36, 742). Although the effect of troglitazone to lower circulating androgens is thought to be mediated by a reduction in plasma insulin, troglitazone has recently been reported to inhibit 3β-HSD, and thus P production, in cultured porcine granulosa-luteal cells (743). The relevance of this finding to troglitazone treatment of women with PCOS remains to be determined.

Taken together, studies of metformin and troglitazone in PCOS suggest that reduction of insulin resistance and hyperinsulinemia leads to a decline in ovarian androgen hypersecretion, lending further support to the hypothesis that insulin resistance and hyperinsulinemia are indeed instrumental in the development of hyperandrogenism in PCOS.

Three other insulin-sensitizing agents are of potential use in PCOS: D-*chiro*-inositol (also called INS-1), β_3-adrenergic receptor agonists, and vanadate (744, 745). D-*chiro*-Inositol, which may serve as a precursor for inositolglycan mediators of insulin signal transduction, has been shown to lower circulating insulin and improve insulin action in spontaneously insulin-resistant primates (744). A recent study suggests that inositolglycans mediate the stimulation of thecal steroidogenesis by insulin (79), and another report suggests that D-*chiro*-inositol, given to women with PCOS in a placebo-controlled trial, decreases insulin secretion during OGTT and increases plasma SHBG. Accompanying these changes was a significant restoration of spontaneous ovulation (746).

β_3-Adrenergic receptors are located in brown fat, a tissue responsible for nonshivering thermogenesis and weight regulation. Ablation of brown adipose tissue in transgenic animals induces insulin resistance (747). When given to obese rodents, β_3-adrenergic receptor agonists can produce weight loss and a reduction in insulin resistance (745).

Vanadate appears to improve insulin action through mechanisms distal to insulin-receptor kinase activation (748–752). Vanadium may activate cytosolic protein tyrosine kinase and thus may mimic the effects of insulin (748, 753). Both in insulin-resistant animals and in those with streptozotocin-induced diabetes, vanadate reduces blood glucose concentration and, in the former group, it reduces circulating insulin levels (745). Vanadyl sulfate can reduce insulin resistance in patients with type 2 diabetes (754).

It remains to be established whether D-*chiro*-inositol, β_3-adrenergic receptor agonists, or vanadate are clinically useful in women with insulin resistance and hyperandrogenism.

B. Therapeutic use of IGF-I and IGF-II

IGF-I has been used therapeutically in several studies in patients with type 1 or type 2 diabetes (755–759), in syndromes of extreme insulin resistance (329, 745, 760), and in myotonic dystrophy and other diseases (761, 762). IGF-I appears to be effective in enhancing the sensitivity of tissues to insulin and in directly inhibiting insulin secretion by pancreatic β-cells (763, 764). Its side effects include symptomatic hypophosphatemia, seen mainly with intravenous administration; arthropathy; and occasional cranial nerve palsies (745).

In patients with syndromes of extreme insulin resistance, injections of IGF-I result in a decline of plasma glucose concomitant with a decrease in insulin and C-peptide (745, 763, 765, 766). The mechanisms of these effects of IGF-I are not well understood. It is possible that in addition to having insulin-like actions of its own mediated by the type I IGF receptor, IGF-I can also indirectly activate the insulin receptor, possibly by initiating insulin receptor phosphorylation (type I IGF receptor/insulin receptor "cross-talk"). Long-term (2-yr) administration of IGF-I to a patient with extreme insulin resistance (type A syndrome) led to a reduction of glucose levels but was associated with worsening of her hyperandrogenism (767). Similarly, prolonged administration of IGF-I to women with GH receptor deficiency is associated with the development of hyperandrogenism (768). To our knowledge, there are no clinical trials of IGF-II in patients with insulin resistance and/or anovulation. When overexpressed in transgenic mice, IGF-II produces improvement in insulin sensitivity and increases in lean body mass without affecting body size (769). The reproductive function of mice overproducing IGF-II has not been examined in detail. Whether IGFs can be safely used in humans with insulin-resistant states, and whether their use will affect ovarian function, awaits further study.

C. Use of GH in ovulation induction

1. GH effects on ovarian function. Another manipulation of the components of the insulin-related ovarian regulatory system that may have therapeutic implications is the use of GH along with gonadotropins in ovulation induction. GH can potentially influence follicular function in four ways: direct action on follicular cells through GH receptors; direct action to increase ovarian IGF production; action on the liver to increase circulating IGF-I; and modulation of intrafollicular and hepatic IGFBP production and/or IGFBP levels in FF and in the circulation.

There is evidence for direct effects of GH on human granulosa cells, which express GH receptors (770–772). GH treatment of granulosa cells *in vitro* stimulates both steroidogenesis and mitogenesis (773–776). Since evidence points against production of IGF-I by human granulosa cells, it is questionable whether GH actions on the human ovary are mediated through ovarian IGF-I. Only the theca layer expresses IGF-I mRNA, but it does not appear to express GH receptors (777). In one study, GH actions on human granulosa cells could be blocked by antibodies to IGF peptides or the type I IGF receptor (775), but in two others (773, 776), IGF-I production by granulosa cells was not detected, even with GH treatment. The latter studies, in addition to those showing that the human ovary, unlike its rodent counterpart, does not produce IGF-I (13, 14, 88, 89), suggest that GH can act directly on granulosa cells through its own receptor. At least one study, however, found no effect of GH on granulosa cell steroidogenesis (778).

GH increases hepatic production of IGF-I, and IGF-I mediates many of the effects of GH. When GH is given on an alternate-day schedule in ovulation induction protocols, both circulating and FF IGF-I levels rise (464, 779–786). The increase of intrafollicular IGF-I most likely mediates the ad-

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juvant effect of GH seen in ovulation induction of some anovulatory patients (see below).

GH treatment added to ovulation induction protocols may also influence intrafollicular IGFBP levels. GH is the principal stimulator of hepatic IGFBP-3 production and hence a major regulator of serum IGFBP-3 (429). In studies of poor responders to conventional gonadotropin ovulation-induction regimens, GH did not affect levels of IGFBP-1 or IGFBP-3 in FF, but it did raise serum IGFBP-3 (461, 464, 784). In a study of unselected women undergoing IVF (786), however, GH raised FF levels of IGFBP-1, -3, and -4, as well as serum levels of IGFBP-3, compared with matched placebo cycles in each subject. The increase in IGFBP-1 likely arises within the follicle, since serum IGFBP-1 is transiently reduced by GH (787). Differences in the IGFBP-3 response among patients suggest that the beneficial effect of increased IGF-I with GH may be blunted by stimulation of IGFBP-3 in some women (786).

The effects of GH treatment on follicular steroidogenesis have also been examined. In COH cycles, FF levels of E_2 and P did not differ between GH- and placebo-treated groups (406, 464, 774, 783, 788, 789). In one study, E_2 and P production by granulosa cells in culture was unaltered by *in vivo* GH exposure (788), but in another, levels of mRNA expression for 3β-HSD and aromatase in freshly harvested granulosa cells were increased by GH (774). Supporting the latter result, a third study found that steroidogenesis by cultured granulosa cells immediately after harvest was increased by *in vivo* GH exposure (775).

There is evidence that endogenous GH secretion may affect ovarian function, particularly in response to gonadotropin stimulation. Women with higher basal GH levels had greater E_2 and oocyte number than those with lower GH levels in one study (790), and in another, serum IGFBP-3 level before stimulation, presumably reflecting integrated GH secretion, was positively correlated with serum E_2 and follicular response to gonadotropins (791). GH reserve, measured by response to a clonidine-provocative test, was lower in poor responders in two studies (792, 793), but not different in a third (794). The GH rise in response to gonadotropin stimulation (795) was predictive of pregnancy, but not of the degree of ovarian stimulation (796). Age and weight may be important confounders, however, as GH levels are lower in women of advanced reproductive age and in obese women (168, 797, 798).

2. Clinical trials of GH in ovulation induction. Given the potential physiological involvement of the GH/IGF-I axis in ovulation, many investigators have attempted to use exogenous GH as an adjuvant for ovulation induction. A preliminary trial by Homburg *et al*. (799) found that GH, in a dose of 20 IU on alternate days, significantly augmented the ovarian response to human menopausal gonadotropins (hMG) in four of seven patients undergoing ovulation induction. The four patients who showed improvement all had hypogonadotropic anovulation. The same group of investigators then undertook a randomized, double-blind, placebo-controlled trial, in which 16 women with hypogonadotropic anovulation were treated with placebo or GH, 24 IU every other day, in addition to hMG. The duration of treatment and the total number of ampules of hMG needed were reduced in the GH group, compared with the placebo group (800). Another report noted a similarly decreased hMG requirement in three anovulatory women, which persisted in the subsequent cycle (801). A study in IVF patients, who were not selected for anovulation but included a majority with ultrasound-demonstrated polycystic ovaries, also found that GH reduced hMG requirement (779). Two other studies, in hypogonadotropic women with polycystic ovaries (802) or clomiphene-resistant women with PCOS (803) undergoing ovulation induction with GnRHa and hMG, found an improvement in hMG response with GH cotreatment. A large, multicenter placebo-controlled study of 64 hypogonadotropic, anovulatory women confirmed that GH decreases the total hMG requirement in a fashion dependent on GH dose, but GH lowered pregnancy rates (804).

In contrast to these results in anovulatory women, studies in ovulatory women undergoing hyperstimulation for IVF have largely failed to show a benefit of adjunctive GH. The largest group of these studies examined poor responders to GnRHa-down-regulated hMG stimulation cycles (779, 784, 789, 805–808). These studies administered GH, typically in a dose of 12 IU on alternate days, concurrently with hMG until adequate follicular maturation was achieved. None demonstrated a statistically significant improvement in pregnancy rate with GH. Of the four studies that employed a double-blind, placebo-controlled design, two (789, 808) found no benefit of GH on cycle stimulation parameters, while two others (779, 784) found a significant improvement with GH only in the fertilization rate. Two studies, however, did note a decreased hMG requirement with GH (779, 805).

GH has also been studied as an adjunct to short (flare) GnRHa-hMG regimens for stimulation before IVF. In a placebo-controlled study of poor responders receiving conventional leuprolide doses, no benefit of GH was found (785). In an open-label study of GH in a microdose (40 μg twice daily) leuprolide flare regimen, follicular development was found to be superior with GH and cycle cancellation avoided in patients whose previous long GnRHa cycles had been canceled (809). In normal responders to hMG or unselected women, no effect of GH was seen on follicular response, oocyte or embryo quality or number, or pregnancy rate in four studies of women undergoing GnRHa-down-regulated hMG treatment for IVF (783, 786, 788, 806).

Given the suggestion of a beneficial effect of GH in some women, particularly those with impaired ovulation, several approaches have been taken to identify candidates for adjunctive GH. In two placebo-controlled studies, women with polycystic ovarian morphology undergoing IVF showed significant improvement with GH in numbers of follicles, oocytes collected, and oocytes fertilized (779, 780). In another approach, blunted responses to provocative tests for GH secretion, which may indicate occult or borderline GH deficiency, have been used to select patients for GH treatment. Anovulatory, nonobese women with decreased GH reserve on a clonidine provocative test showed a 30% lower hMG requirement when given adjunctive GH (792). In another study, conception rates in a mixed IVF/*in vivo* fertilization population were increased by GH in clonidine-nonresponsive, but not in clonidine-responsive, subjects (810). Clonidine-negative women may have dysovulatory features

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similar to those of PCOS, which has been associated with decreased basal GH levels as well as decreased responsiveness to clonidine and L-DOPA (168, 699, 798, 811).

The extensive literature on the use of adjunctive GH in hMG-based ovulation induction regimens has failed to demonstrate the general clinical utility of GH, despite the evidence for an involvement of the GH/IGF-I axis in ovarian follicular function. As noted above, many of the studies had small numbers of subjects, lacked placebo controls, used inconsistent protocols of GH administration, and lacked uniform definitions of "poor responders." These features make comparisons of these studies difficult. At present, given its cost, in our opinion the use of adjunctive GH in all ovulation induction protocols is not warranted. Further large-scale randomized, double-blind clinical trials, which could help determine parameters that allow selection of those patients who will benefit from GH use in ovulation reduction protocols should be conducted.

VII. Summary and Conclusions

In summary, the ovarian insulin-related regulatory system consists of insulin, insulin receptors, IGF-I, IGF-II, type I IGF receptors, type II IGF receptors, IGFBPs 1–5, and IGFBP proteases. There is evidence that the components of this system interact in a complex way (Fig. 7). The insulin-related ovarian regulatory system appears to participate in the regulation of normal ovarian function, including initiation of puberty and ovulation, and its components are altered in certain pathological states, which include type 1 and type 2 diabetes mellitus, obesity, reproductive abnormalities associated with weight loss and starvation, PCOS, and states of extreme insulin resistance. Therapeutic approaches directed toward normalization of the components of this system appear to be promising in some of these diseases.

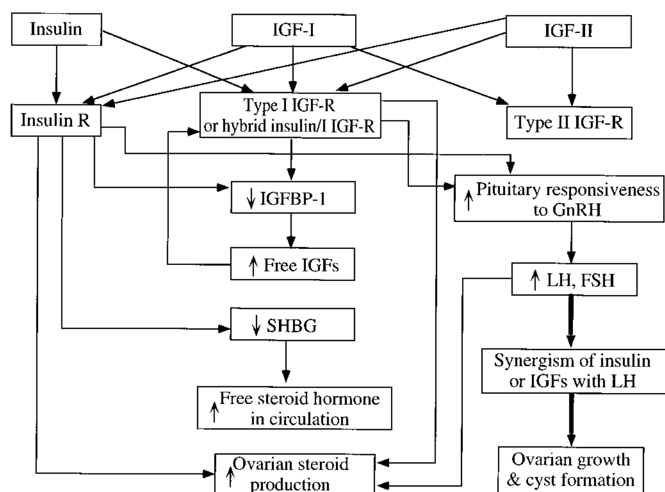

FIG. 7. The relationships among the components of the insulin-related ovarian regulatory system. Insulin, IGF-I, and IGF-II, acting through insulin receptors or type I IGF receptors, increase pituitary responsiveness to GnRH; stimulate gonadotropin secretion directly; stimulate ovarian steroidogenesis; inhibit IGFBP-1 and SHBG production; and act synergistically with gonadotropins to promote ovarian growth and cyst formation (see also Tables 3–5).

Acknowledgments

The authors thank Dr. Steven Spandorfer, Ms. Ming Su, and Ms. Rita Falbel for their assistance in the preparation of the manuscript. The authors apologize to the many investigators who have contributed to the reviewed field but whose work was not cited. Because of the vast literature on the subject, review articles were sometimes quoted instead of the original reports.

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DATE: August 31–September 3, 1999
VENUE: Jerusalem, Israel
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