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Insulin Stimulates Testosterone Biosynthesis by Human Thecal Cells from Women with Polycystic Ovary Syndrome by Activating Its Own Receptor and Using Inositolglycan Mediators as the Signal Transduction System

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ABSTRACT

To determine whether insulin stimulates human ovarian testosterone production in the polycystic ovary syndrome by activating its own receptor and using inositolglycan mediators as the signal transduction system, thecal cells from polycystic ovary syndrome women were isolated and cultured. Insulin and insulin-like growth factor I stimulated thecal testosterone biosynthesis. Antibody blockade of the insulin receptor abolished insulin's stimulatory action, whereas effective antibody blockade of the insulin-like growth factor I receptor did not alter insulin's stimulation of thecal testosterone biosynthesis. A chiro-inositol containing glycan (INS-2) increased thecal testosterone biosynthesis. Preincubation of cells with an antiinositolglycan antibody (A23939 or αIGP) abolished insulin's stimulatory effect, but not that of hCG. These findings suggest that inositolglycans serve as the signal transduction system for insulin's stimulation of human thecal testosterone biosynthesis. (*J Clin Endocrinol Metab* **83:** 2001–2005, 1998)

THE POLYCYSTIC ovary syndrome (PCOS) is a common endocrinopathy that affects approximately 6% of women of reproductive age and is characterized by chronic anovulation and hyperandrogenism (1). Insulin resistance accompanied by compensatory hyperinsulinemia is a common feature of PCOS, and evidence suggests that hyperinsulinemia plays a pathogenic role in causing the hyperandrogenism of the syndrome by increasing ovarian androgen production (2–6). Presumably, this occurs as a result of insulin stimulating testosterone production by the ovarian cell responsible for androgen biosynthesis, namely the thecal cell.

It seems paradoxical that insulin should stimulate ovarian androgen production in a woman who is otherwise resistant to insulin as manifested by decreased glucose utilization (7). Nonetheless, studies in insulin-resistant women suggest that the ovaries remain sensitive to insulin's actions on steroidogenesis, even when classical target organs for insulin (such as muscle, fat, or liver) demonstrate metabolic insulin resistance in terms of decreased glucose disposal (2–6).

It has been proposed that insulin could stimulate ovarian androgen production either by cross-associating with the ovarian insulin-like growth factor I (IGF-I) receptor or by binding to ovarian hybrid insulin receptors (IRs) (7). We believed these explanations to be unlikely because 1) the elevation in circulating insulin in women with PCOS is usually modest, and insulin would not be expected to cross-associate appreciably with the IGF-I receptor; and 2) hybrid IRs have not been identified on human ovaries. We hypothesized that the cellular mechanism by which insulin stimulates ovarian androgen production in PCOS involves activation of a novel signal transduction system that is distinct and separate from the insulin-activated tyrosine phosphate cascade used to enhance glucose utilization. Some actions of insulin involve low mol wt inositolglycan mediators (also known as putative insulin mediators or second messengers) (8–10), and we regarded this putative mediator system as a prime candidate for the alternate signaling mechanism.

To test our hypothesis, we assessed the effects of insulin and IGF-I on testosterone biosynthesis by human thecal cells in the absence or presence of ovarian IR or IGF-I receptor blockade. We also assessed the ability of a synthetic chiro-inositol-containing glycan (INS-2) to enhance thecal testosterone production. INS-2 is a synthetic pseudodisaccharide of pinitol (3-*O*-methyl-D-chiro-inositol) and galactosamine (11) that is believed to be the pH 2.0 D-chiro-inositolglycan putative insulin mediator described by Larner and colleagues (12). Finally, as evidence suggests that inositolglycan mediators are initially formed extracellularly and must subsequently enter the cells to exert their metabolic action (13, 14), we examined the effects of preincubation with two antiinositolglycan antibodies (A23939 and αIGP) on stimula-

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tion of thecal testosterone production by insulin. The results suggest that insulin stimulates human thecal testosterone biosynthesis by activating its own receptor and using inositolglycan mediators as the signal transduction system.

Subjects and Methods

Study subjects

Ovarian tissue was obtained from eight women with PCOS (PCOS cells) and six normally menstruating women (non-PCOS cells). The women were 37–43 yr old and underwent surgery for benign nonovarian gynecological disease. They had not received any hormonal medication for at least 4 months before surgery. Subject characteristics are shown in Table 1. The procurement and use of human ovarian tissue was approved by the local institutional review board, and informed consent was obtained from each woman.

Women with PCOS had oligomenorrhea (fewer than six menstrual periods in the last year), an elevated serum free testosterone concentration, and ovarian ultrasonic findings consistent with the diagnosis of PCOS (15). Normal women menstruated regularly every 28–30 days and had normal serum free testosterone concentrations and normal ovaries on ultrasonic examination. Ovaries were classified as polycystic by macroscopic morphological features at the time of dissection. An ovary was defined as polycystic if it contained 10 or more follicles, each 2–8 mm in diameter, which were distributed peripherally, and demonstrated at least one of the following three histological features: 1) increased density and amount of ovarian stroma, 2) increased ovarian volume (>9 mL), or 3) thickened tunica albicans.

Thecal cell dissection

Resected ovarian tissue was immediately placed in ice-cold serum-free culture medium, which was also used for follicular dissection, consisting of medium 199 containing Hanks' salts and supplemented with 25 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B (as fungizone in 0.85% saline; all reagents obtained from Life Technologies, Grand Island, NY), and transported to the laboratory.

Ovarian specimens were washed twice in fresh medium, and gross morphological features were recorded. Individual follicles more than 4 mm in size were isolated from ovarian stroma by dissection using zoom stereomicroscopy. Follicular fluid was aspirated using a microsyringe, and the follicle wall was cut open with microscissors. After scraping all visible granulosa cells from the lamina basalis, the hemisected theca internae from several follicles were rinsed twice in fresh medium.

Mechanical dispersion of thecal cells was performed by a modification of the techniques described by Richards and Kersey (16) and Yunis (17). Isolated theca interna sheets were chopped, minced finely, and gently shaken for 20 min in a shaking water bath at 37 C. This was repeated twice, and final dispersion was achieved and confirmed stereomicroscopically by six to eight repeat aspirations through a Pasteur pipette followed by gentle mixing. Cells were pelleted by centrifugation at 2000 rpm for 10 min, resuspended in fresh medium, and washed twice more. The thecal cell pellet was resuspended in fresh medium. At least 85% of the thecal tissue (by weight) was dispersed into a suspension of individual cells that was adjusted to a final cell count of 10^6 cells/mL. Total cell counts were performed with a hemocytometer; cell viability, as assessed by trypan blue exclusion, was greater than 90%.

TABLE 1. Clinical characteristics of women

All women with PCOS had oligo- or amenorrhea and elevated serum free testosterone levels, whereas non-PCOS women had regular monthly menses and normal serum free testosterone levels.

Subject no.	Age (yr)	Wt (kg)	Ht (m)	Ovarian ultrasound	Diameter of largest follicle (mm)
Women with PCOS					
1	43	67	1.72	PCOS	11
2	40	87	1.65	PCOS	8
3	38	84	1.62	PCOS	18
4	40	78	1.60	PCOS	12
5	41	59	1.50	PCOS	14
6	37	68	1.58	PCOS	16
7	38	91	1.62	PCOS	14
8	38	87	1.61	PCOS	14
Non-PCOS women					
9	39	50	1.60	Normal	6
10	40	56	1.64	Normal	4
11	38	58	1.60	Normal	8
12	40	65	1.70	Normal	7
13	37	56	1.66	Normal	8
14	41	62	1.60	Normal	4

Thecal cell culture

Thecal cells (10^6 cells/mL) were incubated in 35.7 × 17.3-mm wells (Nunclon, Naperville, IL) containing serum-free culture medium in humidified air and 5% CO_2 at 37 C for up to 144 h. Cells were incubated in the absence of additions or in the presence of insulin (Humulin, Eli Lilly Co., Indianapolis, IN), IGF-I (R&D Systems, Minneapolis, MN), hCG (Profasi, Serono, Italy), or INS-2 (Insmed Pharmaceuticals, Richmond, VA) in the concentrations indicated in the figure legends. In some experiments a well characterized murine antihuman IR (anti-hIR; provided by Dr. Richard Roth, Stanford University, Stanford, CA) (18), murine monoclonal anti-human IGF-I receptor antibody (αIR3; Calbiochem, Oncogene Research Products, Manhasset, NY), nonimmune rabbit IgG (Pierce, Rockford, IL), rabbit polyclonal antiinositolglycan antibody A23939 (Insmed Pharmaceuticals), or antiinositolglycan antibody αIGP (courtesy of Dr. Joseph Larner, University of Virginia, Charlottesville, VA) was added to the incubation medium 15 min before treatment with insulin, INS-2, or hCG.

Incubations were terminated by rapid freezing in an acetone-dry ice bath and stored at −20 C until assayed. The testosterone content of cells and media were analyzed in duplicate by RIA (Diagnostic Systems Laboratories, Webster, TX), with intra- and interassay coefficients of variation of 4.5% and 9.8%, respectively.

Preparation of antiinositolglycan antibodies A23939 and αIGP

A23939 is a polyclonal specific hyperimmune serum directed against INS-2 that was raised in rabbits to the native INS-2 molecule using a complex comprised of INS-2 covalently bound to BSA via a succinyl linkage. The αIGP antibodies were raised against α-galactosidase-treated variant surface glycoproteins from *Trypanosoma brucei* and purified as described previously (14). A complete description of these antibodies has been published (14). They have been shown to bind selectively to the inositolglycan moiety of the glycosylphosphatidylinositol anchor of *T. brucei* variant surface glycoproteins, to inhibit the action of purified insulin mediators *in vitro*, and to block selectively some of the metabolic effects of insulin on BC_3H1 murine myocytes (14).

Results

Stimulation of testosterone biosynthesis by insulin and IGF-I

In time-course studies, insulin stimulated testosterone biosynthesis in both PCOS and non-PCOS cells by 6–9 h of incubation, with peak stimulation between 12–16 h and a slow decline after 48 h (data not shown); therefore, all subsequent experiments were conducted using 16-h incubations. Insulin (20 μg/mL) treatment of thecal cells isolated from the eight women with PCOS resulted in a 13-fold increase in testosterone biosynthesis (control, 1.2 ± 0.4; insulin, 13.2 ± 1.4 pg/10^6 cells·16 h; $P < 0.0001$). Insulin also stimulated testosterone biosynthesis by thecal cells from the six non-PCOS women, but the magnitude of the response was only

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about a fourth of that observed in PCOS cells (non-PCOS, 3.7 ± 1.5; PCOS, 13.2 ± 1.4 pg/10^6 cells·16 h; $P < 0.005$).

In dose-response studies, insulin stimulation of thecal testosterone biosynthesis was demonstrable in PCOS cells at a dose of 2–5 μg/mL (Fig. 1). The dose-response curve for cells from non-PCOS women demonstrated a lower maximal production compared to cells from women with PCOS (Fig. 1), suggesting decreased responsiveness in non-PCOS cells.

Blockade of the IR by anti-hIR

To determine whether insulin stimulates thecal testosterone production by activating its homologous receptor, PCOS cells were preincubated with a well characterized anti-hIR monoclonal antibody that acts as an antagonist for insulin-stimulated responses in human cells (18) before exposing the cells to insulin. In two separate experiments that used cells from different women, blockade of the IR almost totally prevented stimulation of testosterone biosynthesis by insulin, whereas stimulation by the unrelated peptide hCG was unaltered. The results from one of these experiments are illustrated in Fig. 2.

Blockade of the IGF-I receptor by αIR3

IGF-I, at a concentration of 100 ng/mL, maximally stimulated thecal testosterone biosynthesis in both PCOS cells (Fig. 3) and non-PCOS cells (data not shown) and did so to a degree similar to that of insulin.

To determine whether the presence of a functional IGF-I receptor is necessary for insulin to stimulate thecal testosterone production, PCOS cells were preincubated with the monoclonal anti-IGF-I receptor antibody αIR3 before exposure to insulin or IGF-I. Blockade of the IGF-I receptor completely prevented stimulation of testosterone biosynthesis by IGF-I, whereas insulin treatment continued to exert its stimulatory action (Fig. 3).

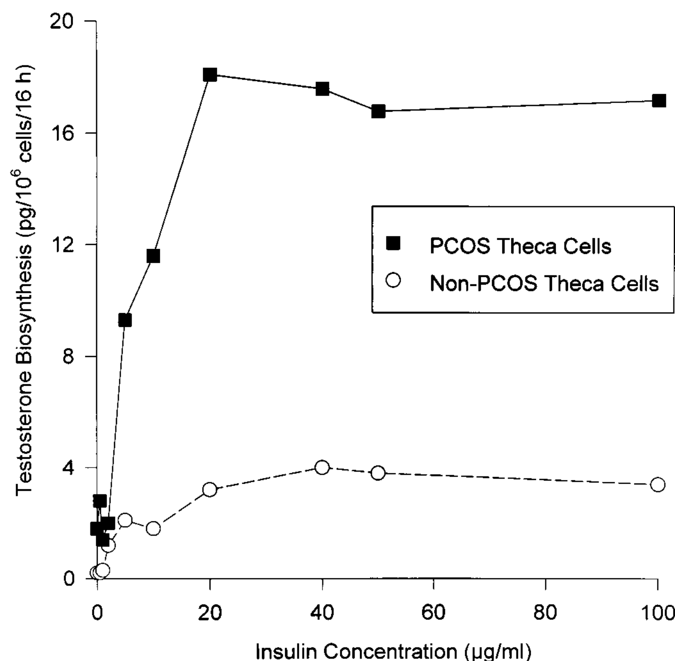

FIG. 1. Dose-response study of insulin's stimulation of testosterone biosynthesis in PCOS and non-PCOS thecal cells. Thecal cells were isolated from one woman with PCOS (PCOS) and one healthy woman (non-PCOS) and cultured in the absence or presence of various concentrations of insulin. After 16 h, cells were quickly frozen and thawed, and the testosterone content of cells and media was assayed by RIA. Net steroid synthesis was calculated by subtracting the steroid content of cells and media at time zero.

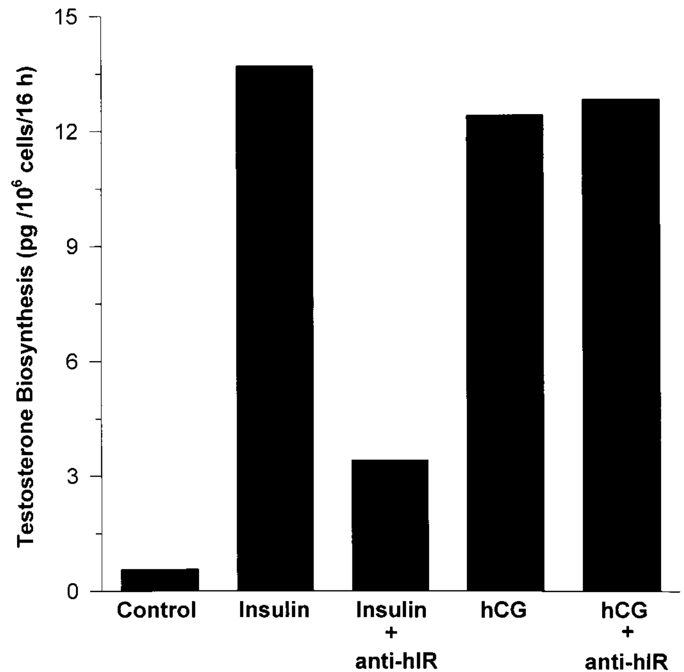

FIG. 2. Effect of preincubation of PCOS thecal cells with anti-hIR on stimulation of testosterone biosynthesis by insulin. Thecal cells from a woman with PCOS were cultured in the absence or presence of insulin (20 μg/mL) or hCG (50 ng/mL). Before treatment, some incubates were preincubated with anti-hIR (10 μg/mL), a monoclonal anti-hIR antibody, at a titer of 1:1000 for 10 min. Incubations were terminated after 16 h and processed as described in Fig. 1.

Stimulation of testosterone biosynthesis by INS-2

A synthetic chiro-inositol-containing glycan, INS-2 (100 μmol/L), consistently stimulated testosterone production by PCOS thecal cells to a degree equal to or greater than that of insulin in five separate experiments, and in a dose-response study INS-2 stimulated testosterone production by PCOS cells at a concentration as low as 1.0 μmol/L (Fig. 4).

Effects of the neutralizing antiinositolglycan antibody A23939 or αIGP

To examine further the possibility that inositolglycan mediators are the signal transduction system for insulin's stimulation of testosterone production by PCOS thecal cells, the polyclonal antiinositol antibody A23939 was employed. In two separate experiments, PCOS thecal cells were preincubated with either nonimmune rabbit Ig (rbIgG; 100 μg/mL) or A23939 (100 μg/mL) and then treated with maximally effective concentrations of insulin. Neither rbIgG nor A23939 alone influenced testosterone biosynthesis (data not shown). As shown in Fig. 5, insulin treatment increased testosterone biosynthesis by almost 9-fold ($P < 0.05$), and preincubation

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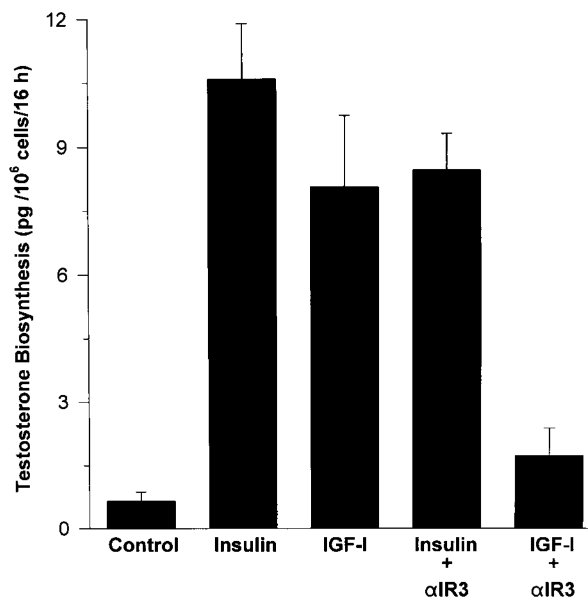

FIG. 3. Effect of preincubation of PCOS thecal cells with αIR3 on stimulation of testosterone biosynthesis by insulin or IGF-I. Thecal cells from four women with PCOS were each cultured separately in the absence or presence of insulin (20 μg/mL) or IGF-I (100 ng/mL). Before treatment, some incubates were preincubated with αIR3 (10 μg/mL), a monoclonal anti-IGF-I receptor antibody, for 10 min. Incubations were terminated after 16 h and processed as described in Fig. 1. Values represent the mean ± SE of four ovaries in each group.

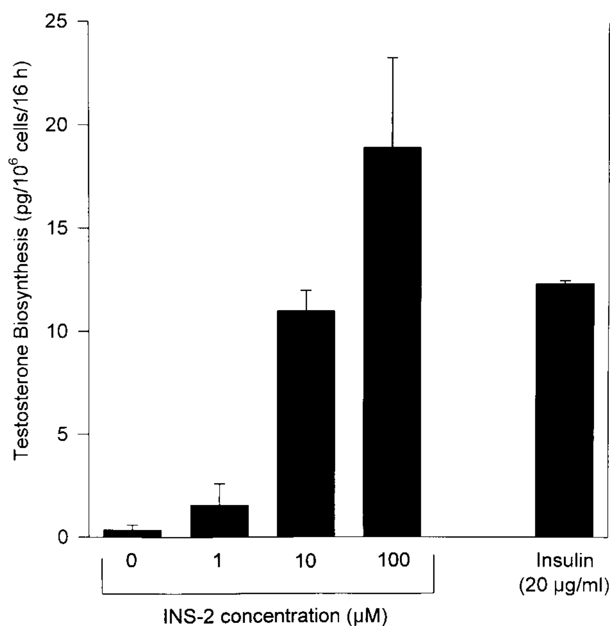

FIG. 4. Dose-response study of INS-2-stimulated testosterone biosynthesis in PCOS thecal cells. Thecal cells from two women with PCOS were each cultured separately in the absence or presence of the indicated concentrations of INS-2 or insulin. Incubations were terminated after 16 h and processed as described in Fig. 1. Values represent the mean ± range.

with rbIgG did not alter insulin's stimulatory effect. Preincubation with A23939, however, totally abolished insulin's ability to stimulate testosterone biosynthesis. Similarly, INS-2 treatment stimulated testosterone biosynthesis by

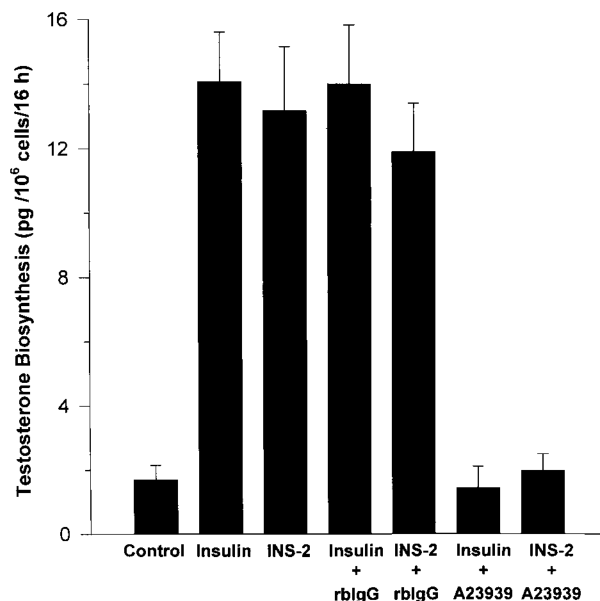

FIG. 5. Effect of preincubation of PCOS thecal cells with A23939 or nonimmune rbIgG on stimulation of testosterone biosynthesis by insulin or INS-2. Thecal cells from six PCOS women were each cultured separately in the absence or presence of insulin (20 μg/mL) or INS-2 (100 μmol/L). Before treatment, some incubates were preincubated with A23939 (100 μg/mL), a polyclonal antiinositolglycan antibody, or nonimmune rbIgG (100 μg/mL) for 10 min. After 16 h, cells were quickly frozen and thawed, and the testosterone content of cells and media was assayed by RIA. Net steroid synthesis was calculated by subtracting the steroid content of cells and media at time zero. Values represent the mean ± SE of six ovaries in each group.

8-fold ($P < 0.05$); INS-2's stimulatory effect was not altered by preincubation with rbIgG, but was completely prevented by preincubation with A23939.

To serve as a control and demonstrate that A23939's inhibitory effect was specific for insulin, the effect of preincubation with A23939 (100 μg/mL) on stimulation of testosterone biosynthesis by hCG was examined. hCG (50 ng/mL) treatment increased testosterone biosynthesis by PCOS cells by 7-fold (control, 1.3 ± 0.4 pg/10^6 cells·16 h; hCG, 9.2 ± 0.5 pg/10^6 cells·16 h; $P < 0.05$), and this stimulatory effect was not altered (P = NS) by preincubation with A23939 (hCG plus A23939, 9.6 ± 1.0 pg/10^6 cells·16 h).

To confirm the results of the A23939 experiment, a second antiinositolglycan antibody, αIGP, was employed. αIGP has been well characterized and is known to block the action of inositolglycan mediators (14). Preincubation of cells from one woman with PCOS with αIGP (400 μg/mL) abolished the ability of insulin (20 μg/mL) to stimulate testosterone biosynthesis by thecal cells (control, 0.03 pg/10^6 cells·16 h; insulin, 14.8 pg/10^6 cells·16 h; insulin plus αIGP, 1.3 pg/10^6 cells·16 h).

Discussion

The results of these studies confirm those previously reported that insulin stimulates testosterone biosynthesis by human thecal cells (13, 14, 19). They also demonstrate that insulin stimulates testosterone biosynthesis by thecal cells from both PCOS and non-PCOS ovaries. The findings sug-

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gest that the cells from non-PCOS ovaries may be less responsive to insulin than those from PCOS ovaries. However, caution should be exercised when interpreting these results, because differences in the sizes of the isolated follicles (4–8 mm from non-PCOS women and 11–18 mm from PCOS women) leave open the possibility that some of the follicles from PCOS women represented dominant follicles, which would be expected to possess enhanced androgen-synthesizing capacity. Nonetheless, these findings are consistent with the clinical observation that hyperinsulinemia resulting from obesity appears to increase ovarian testosterone production in women with PCOS, but not in non-PCOS women (6, 20).

Our studies indicate that insulin's stimulation of testosterone biosynthesis by PCOS thecal cells is obviated by blockade of the IR, but is unaltered by effective blockade of the IGF-I receptor. This suggests that insulin's action is mediated by binding to and activation of its homologous receptor and is consistent with similar findings in human granulosa cells (21).

Most notably, these studies demonstrate that inositolglycan mediators probably serve as the signal transduction system for insulin's stimulation of human thecal testosterone biosynthesis. In support of this idea, a synthetic chiro-inositol-containing glycan (INS-2) mimicked insulin's stimulation of thecal testosterone biosynthesis in a concentration-dependent manner and to a degree at least equal to that of insulin.

Further evidence supporting this idea resulted from studies employing the polyclonal antiinositolglycan antibodies A23939 and αIGP. Inositolglycan mediators reside on the cell surface, and it had been previously demonstrated that antiinositolglycan antibodies bind to insulin mediators released by insulin from BC_3H1 cells and prevent their cellular uptake (14). Preincubation of thecal cells with A23939 or αIGP abolished the ability of maximally effective concentrations of insulin to stimulate testosterone biosynthesis. Specificity of inhibition was demonstrated by showing that preincubation of cells with A23939 did not alter hCG-stimulated testosterone biosynthesis. Collectively, these findings lend support to the idea that some insulin mediators are generated outside the cell (13, 14) and further suggest that insulin mediators constitute a significant signaling mechanism for the transduction of insulin's stimulation of human ovarian androgen production. They are also consistent with reports that the inositolphosphoglycan second messenger system transduces other steroidogenic actions of insulin in human cytotrophoblasts (22) and swine granulosa cells (23).

The importance of these observations is that the inositolphosphoglycan signal transduction system may remain intact and fully functional in conditions otherwise characterized by insulin resistance in terms of a defective tyrosine kinase cascade system leading to impaired glucose transport and utilization. By using the alternate inositolglycan signal transduction pathway, insulin's stimulation of ovarian androgen biosynthesis is preserved even in the face of glucose intolerance. Therefore, these findings yield an explanation for the seeming clinical paradox that hyperinsulinemia stimulates ovarian androgen production in women with PCOS, even while these women are resistant to insulin's enhancement of peripheral glucose transport or utilization.

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