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The insulin resistant subphenotype of polycystic ovary syndrome: Clinical parameters and pathogenesis

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KEY WORDS
Polycystic ovary syndrome
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Insulin receptor
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Objective: This study was undertaken to compare clinical and biochemical characteristics of the insulin resistant (IR) and non-IR subphenotypes of polycystic ovary syndrome (PCOS).
Study design: Infertile PCOS women were classified as IR (n = 32) or non-IR (n = 46) on the basis of fasting glucose and insulin levels. The incidence of acanthosis nigricans (AN), hirsutism, and ovulation in response to clomiphene citrate (CC) was compared between the 2 groups, along with serum levels of gonadotropins, and sex steroids. Blood samples from 28 PCOS patients and 8 controls were analyzed by enzymatic immunoassay for autophosphorylated insulin receptor (APIR) and total insulin receptor (TIR) content.
Results: Insulin resistance was associated with obesity (odds ratio [OR] = 3.5, $P<.05$), AN (OR = 6.0, $P<.05$), hirsutism (OR = 3.1, $P<.05$), and resistance to CC (OR = 5.0, $P<.05$). Mean levels of LH, LH/FSH ratios, and testosterone were lower in women with IR (11.5 ± 6.8 mIU/mL, 2.0 ± 1.0, and 56.6 ± 29.0 ng/dL, respectively) compared with women without IR (15.0 ± 13.4 mIU/mL, 2.4 ± 1.5, and 72.5 ± 29.8 ng/dL, respectively) ($P<.05$). Mean APIR/TIR ratios in IR women were lower than in non-IR women ($P<.05$ at 100 nmol/L of insulin) and controls ($P<.01$ at 1, 10 and 100 nmol/L insulin).
Conclusion: Patients with IR are more likely to be obese and have AN, hirsutism, resistance to CC, and lower LH, LH/FSH ratios, and testosterone levels. Furthermore, IR patients appear to have defective autophosphorylation of the insulin receptor, a key element in insulin action, and a possible mechanism for IR in PCOS.

Polycystic ovary syndrome (PCOS) affects 4% to 6% of women of reproductive-age and is a common cause of female infertility.[1] The syndrome is characterized by hyperandrogenic chronic anovulation; recently, insulin resistance (IR) has been recognized as a significant finding.[2]

IR and compensatory hyperinsulinemia may explain several clinical and biochemical findings in PCOS. Hyperinsulinemia may contribute to hyperandrogenemia

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by direct effect on the ovary,[3] as well as by a negative impact on sex hormone-binding globulin (SHBG) levels.[4] Hyperinsulinemia may also lead to impaired lipolysis in adipocytes, which in turn may contribute to obesity often seen in PCOS patients.[5,6] Furthermore, the mitogenic effects of insulin on basal cells of the skin may result in acanthosis nigricans (AN),[7] which can be present in PCOS patients. Taken together, such insulin effects have made IR and hyperinsulinemia an attractive theory behind the pathophysiology of PCOS. In fact, it has been suggested by some investigators that IR is present in all PCOS patients.[8] However, others have reported that IR is not a universal finding, but rather is present in no more than 50% to 70% of PCOS patients.[9] This suggestion is supported by the observation that the use of insulin-sensitizing medications such as metformin, results in a limited overall clinical response in PCOS patients.[10,11] Therefore, IR may represent an important factor in a proportion of PCOS patients, rather than an essential component of the syndrome.

A ratio of fasting glucose (G) to fasting insulin (I) has been qualified as a simple and useful predictor of insulin resistance in women with PCOS.[12] Other, more invasive, tests such as insulin-glucose clamp studies, intravenous glucose tolerance tests, and quantitative model indices, may also diagnose insulin resistance[9]; however, none of the tests describe pathologic IR at the cellular level. In 1992, Ciaraldi et al[13] described decreased insulin-stimulated autophosphorylation of the insulin receptor (APIR) in adipocytes isolated from women with PCOS. Dunaif et al,[14] studying cultured adipocytes and myocytes, proposed reduced insulin receptor tyrosine kinase activity secondary to excessive serine phosphorylation as a potential mechanism for IR in PCOS. Insulin binding and APIR had previously been characterized in human erythrocytes using cumbersome gel electrophoresis and autoradiography techniques.[15,16] In 1994, Hagino et al[17] introduced a sensitive enzyme-linked immunosorbent assay (ELISA) for detection of APIR and total insulin receptor (TIR) in erythrocytes isolated from whole blood. To typify the clinical features of IR in PCOS, we compared common clinical and laboratory characteristics among IR and non-IR PCOS patients. Furthermore, to examine previously proposed mechanisms for IR in PCOS, we adapted the laboratory methods of Hagino et al[17] to compare APIR activity between IR and non-IR PCOS patients.

Material and methods

The study received Institutional Review Board approval. Between January 2000 and July 2002, women who presented to the Reproductive Endocrinology and Infertility Clinic at the University of Southern California with an initial complaint of infertility were diagnosed with PCOS on the basis of the following criteria: chronic anovulation ($\leq$6 menstrual episodes per year), hyperandrogenemia (total testosterone [T] $\geq$60 ng/dL) or clinical hyperandrogenism as evidenced by hirsutism (Ferriman-Gallwey score $\geq$8),[18] and/or severe persistent acne, and exclusion of adrenal hyperplasia (17-OH progesterone <200 ng/dL), hyperprolactinemia (prolactin <25 ng/mL), and hypothyroidism (thyroid stimulating hormone <5.0 μIU/mL). Patients receiving metformin were excluded from the study. Blood samples were obtained after an overnight fast for measurement of G and I, and subjects were categorized into 2 groups, based on the presence or absence of IR (fasting G/I <4.5, and/or fasting I level $\geq$20 μIU/mL).[12] A total of 78 patients were enrolled in the study.

Biochemical and clinical parameters

Blood samples were obtained for measurement of luteinizing hormone (LH), follicle-stimulating hormone (FSH), dehydroepiandrosterone sulfate (DHEAS), prolactin, and estradiol (E2) concentrations, by means of immunoassays, that used commercially available kits. Testosterone was measured by manual radioimmunoassay (Coat-a-Count, Diagnostic Products Corp, Los Angeles, Calif). Fasting G/I ratios, LH/FSH ratios, and body mass indices (BMIs) [weight (kg)/height $(m)^2$] were calculated. Obesity was defined as BMI $\geq$ 30 kg/m^2. The presence or absence of AN, as evidenced by characteristic velvety skin discoloration on the neck, axilla, or groin regions,[7] and hirsutism, was determined during the initial visit, before obtaining results of fasting glucose and insulin levels.

A subset of 44 women underwent ovulation induction using clomiphene citrate (CC). An initial dose of 50 mg of CC was started on day 5 of progesterone-induced menses and administered for 5 consecutive days. Ovulation was determined by a midluteal serum progesterone level 3 ng/mL or more on day 23 of the cycle, or by a positive serum ß-hCG. Serum progesterone and/or ß-hCG levels were available in all 44 patients. Patients who failed to ovulate or conceive received CC at incremental doses of 50 mg, up to a maximum daily dose of 250 mg.

APIR testing

Patients signed informed written consent before participation in APIR testing. Twenty-eight PCOS patients (IR [n = 13], non-IR [n = 15]), and 8 healthy controls of reproductive-age with regular menstrual cycles, underwent APIR testing. Patients were not receiving any medication for a minimum of 30 days before testing. Whole blood was obtained from all patients after an 8-hour overnight fast. Testing for APIR was performed according to the following protocol: The APIR was quantified by ELISA as described previously.[17]

Table I Characteristics of 78 PCOS patients

	Age (y)	Gravidity	Parity	Avg menses interval(mons)	Weight (kg)	BMI (kg/m^2)	Obese %	AN %	Hirsute %	Ovulation %
Non-IR group (n=46)	27 ± 4	0.4 ± 0.7	0.2 ± 0.4	3.0 ± 1.8	75.2 ± 14.1	30.0 ± 4.6	39	4	32	54(n=28)
IR group (n=32)	28 ± 5	0.4 ± 0.7	0.2 ± 0.5	3.7 ± 2.4	82.3 ± 20.9	35.0 ± 8.0	72	22	58	19(n=16)
P value	NS	NS	NS	NS	NS	<.05	<.05	<.05	<.05	<.05

Table II Comparison of biochemical and clinical parameters between IR and non-IR PCOS patients

	G mg/dL	I μU/mL	G/I ratio mg/10^{-4}U	LH mIU/mL	FSH mIU/mL	LH/FSH	E_2 pg/mL	T ng/dL	DHEAS μg/dL
Non-IR group (n=46)	84.5 ± 12.2	10.7 ± 4.4	9.4 ± 4.6	15.0 ± 13.4	5.8 ± 1.6	2.4 ± 1.5	52 ± 2	72.5 ± 29.8	238 ± 94
IR group (n=32)	93.2 ± 13.8	30.5 ± 9.5	3.3 ± 0.8	11.5 ± 6.8	5.6 ± 1.2	2.0 ± 1.0	53 ± 53	56.6 ± 29.0	212 ± 109
P value	<.05	<.05	<.05	<.05	NS	<.05	NS	<.05	NS

Expressed in means (± SD) and proportions.

Heparinized blood was collected from each subject. The erythrocytes were washed, centrifuged, and resuspended in medium with various concentrations of insulin (0, 1, 10, 100 nmol/L). The cells were then incubated for 15 minutes at 37°C to induce APIR. The reaction was stopped by adding 16 mL of 5 mmol/L Tris-HCl (pH 8.0) containing 2 mmol/L sodium orthovanadate, 1 mg/mL bacitracin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 mmol/L EDTA. After centrifugation at 28,000×*g* for 30 minutes, the supernatants were discarded and the erythrocyte ghosts were resuspended in a HEPES buffer, after which they were centrifuged at 100,000×*g* for 15 minutes at 4°C. The supernatants were discarded and the pellets were solubilized with Triton X-100, and centrifuged at 100,000×*g* for 15 minutes at 4°C; the resulting supernatants were stored at −80°C until analysis.

APIR standard was prepared from Chinese Hamster Ovarian T cells, which overexpress the insulin receptor. After preincubating the cells for 30 minutes at 37°C with serum-free/Ham's F-12 medium, and treating the cells with 10^{-7} mol/L insulin for 10 minutes, the cells were lysed with Triton X-100. After 40 minutes on ice, the lysate was centrifuged at 100,000×*g* for 15 minutes at 4°C, and the supernatant was aliquoted and stored at −80°C.

Quantification of APIR and total insulin receptor (TIR) in the erythrocyte lysates was determined by comparison to the respective standards, using ELISAs on separate microliter plates. For both APIR and TIR determination, plates were coated with monoclonal insulin receptor antibody (Dr Richard Roth, Stanford University, Palo Alto, CA) and washed with buffer. To minimize nonspecific binding, a blocking buffer was added to the wells and the plates were incubated for 30 minutes at 37°C. After washing the plates, 100 μL of each sample and appropriate standard were added to the wells and the plates were incubated overnight at 4°C, and washed again. To quantify APIR, biotinylated autophosphotyrosine antibody was added (Dr Richard Roth, Stanford University, Palo Alto, Calif), and the plates were incubated for 2 hours at room temperature. This step was followed by addition of peroxidase-conjugated streptavidin, and the peroxidase activity was determined colorimetrically at 405 nm. The ELISA for TIR was carried out in the same manner as for APIR, with the exception that a peroxidase conjugated anti-insulin receptor monoclonal antibody was used. In this manner, the APIR/TIR ratio was determined for each sample.

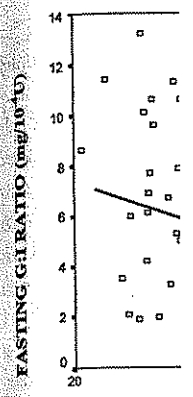
Statistical analysis

Data were analyzed with SPSS software (Statistical Package for the Social Sciences, version 10.0; SPSS, Inc, Chicago, Ill). Student *t* test was used for comparison of means. Analysis of variance was applied for multiple comparisons of means (APIR/TIR ratios). The χ^2 analysis was used for comparison of proportions. Pearson's correlation analysis was used when applicable. Significance was determined at $P<.05$.

Results

Patient characteristics and clinical findings

Of the 78 PCOS patients included in the study, 32 women (41%) had IR and 46 women (59%) were classified as non-IR. Of the 32 IR patients, 28 (87.5%) had both a G/I ratio less than 4.5 mg/10^{-4}U and a fasting I 20 μU/mL or greater, whereas 2 patients (6.25%)



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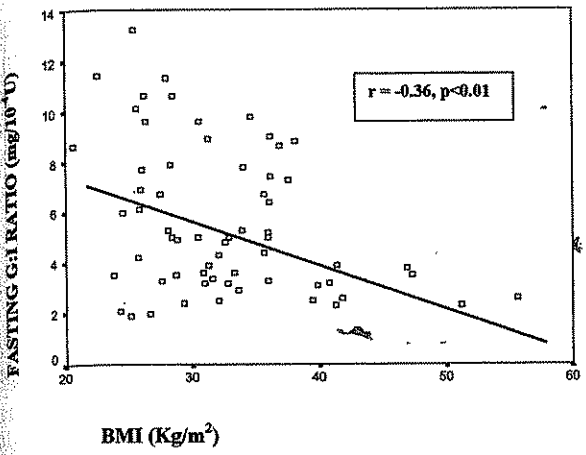

Figure 1 Correlation of BMI and fasting G/I ratio in 78 PCOS patients.

had only an abnormal G/I ratio, and 2 patients only had an abnormal fasting I level. Patient characteristics were similar between the 2 groups, except for mean BMI, which was significantly higher in patients with IR than in patients lacking IR (Table I). A significant association was observed between IR and obesity (OR = 3.5, $P<.05$), AN (OR = 6.0, $P<.05$), and hirsutism (OR = 3.1, $P<.05$) (Table I).

Ovulation induction

Of the 44 PCOS patients who underwent ovulation induction with CC, 16 patients had IR, whereas 28 patients were non-IR. The proportion of patients who ovulated in response to CC was significantly higher in patients lacking IR (54%) compared with patients with IR (19%) (OR = 5.0, $P<.05$), despite similar BMI (33.8 ± 7.8 kg/m^2 vs 30.0 ± 4.7 kg/m^2, P = NS), and mean maximum CC dose used (100 ± 61 mg vs 98 ± 52 mg, P = NS), in IR and non-IR patients, respectively.

Biochemical parameters

By design, mean fasting G, I, and G/I ratios differed significantly between women with IR and women without IR (Table II). Across the study population, a significant inverse correlation was observed between BMIs and fasting G/I ratios ($r = -0.36$, $P<.01$) (Figure 1). A comparison of measured biochemical parameters is depicted in Table II.

APIR testing

Among the 36 women who underwent APIR testing, mean age, gravidity, and parity were similar between IR PCOS women (n = 13), non-IR PCOS women (n = 15), and controls (n = 8). Mean BMI in IR PCOS patients (38.1 ± 6.9 kg/m^2) was significantly higher than mean BMI in non-IR PCOS patients (32.3 ± 6.2 kg/m^2),

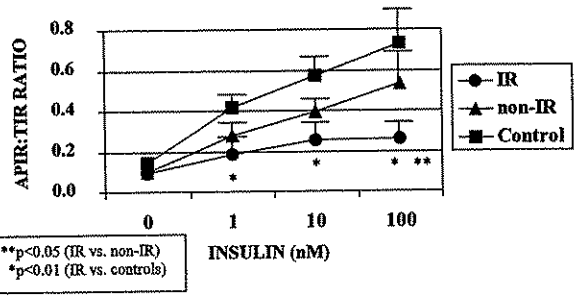

Figure 2 Mean APIR/TIR ratios at different insulin concentrations in IR (n = 13), non-IR (n = 15), and controls (n = 8).

who had a significantly higher BMI than controls (24 ± 4 kg/m^2). As observed in the study population as a whole, mean fasting G/I ratios were lower in IR compared with non-IR PCOS patients who had APIR testing (data not shown). Results for mean APIR/TIR ratios in the 3 groups are depicted in Figure 2. Mean APIR/TIR ratios in IR women were lower than in non-IR ($P<.05$ at 100 nmol/L of I) and controls ($P<.01$ at 1, 10, and 100 nmol/L of I), whereas mean APIR/TIR ratios in non-IR women did not differ significantly from controls. In IR women, APIR/TIR ratios reached a plateau at insulin concentrations above 10 nmol/L. In IR patients, mean APIR/TIR ratios correlated positively with mean fasting G/I ratios ($r = +0.62$, $P<.05$) (Figure 3). As in the group as a whole, BMI correlated inversely with fasting G/I ratio ($r = -0.5$, $P<.05$). An inverse correlation was also observed between BMI and APIR/TIR ratio (100 nmol/L) ($r = -0.46$, $P \leq .05$) (Figure 4).

Comment

Insulin resistance and compensatory hyperinsulinemia are commonly found in PCOS patients; however, not all PCOS patients are affected. By using the euglycemic clamp technique, Dunaif et al[19] demonstrated reduced insulin action in 11 of 29 (38%) of PCOS patients. Legro et al,[12] using the frequently sampled intravenous glucose tolerance test, demonstrated IR in 53% of non-Hispanic obese PCOS patients. Consistent with prior studies, in the current study, only 41% of PCOS patients were found to have IR, whereas 59% demonstrated no IR, as determined by fasting glucose and insulin levels. Designating PCOS patients as either IR or non-IR allowed comparison of common parameters between the 2 groups, in an attempt to identify the clinical consequences of IR and hyperinsulinemia.

Although IR may be found in both obese and lean PCOS patients,[19,20] obesity is a well-described risk factor for IR.[21,22] Consistent with this, in the current study, BMI correlated inversely with fasting G/I ratio, suggesting that in PCOS patients, IR worsens with the degree of obesity. As expected, mean BMI in our IR patients was significantly higher than in non-IR patients, and

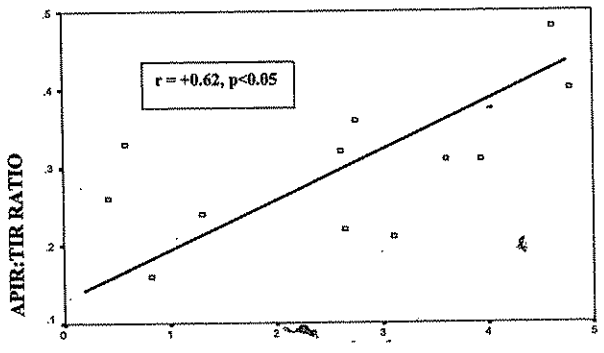

Figure 3 Correlation of fasting G/I ratio with APIR/TIR ratio at 100 nmol/L insulin concentration in insulin resistant PCOS patients.

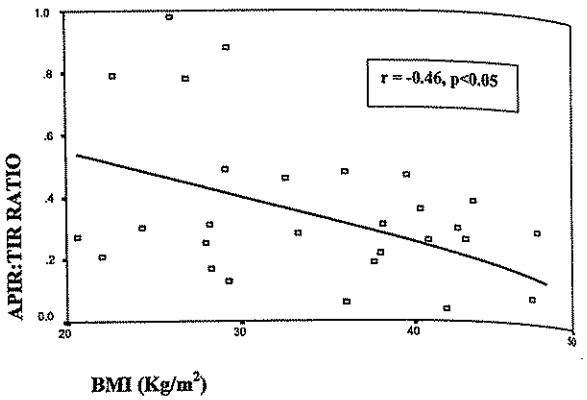

Figure 4 Correlation of BMI and APIR/TIR ratio at 100 nmol/L insulin concentration in patients who underwent APIR testing.

the prevalence of obesity in IR patients (72%) was significantly higher than that in non-IR patients (39%). The association between IR and obesity is not an incidental one, as insulin in excess may itself stimulate central adiposity, which, in turn, exacerbates IR.[6]

Insulin exerts a mitogenic effect on basal cells of the epidermis that can lead to AN, a condition of hyperkeratosis and increased skin pigmentation often observed in the axilla, neck, and groin regions. This skin manifestation is a specific clinical indicator of IR.[7] Not surprisingly, we found a significant association between AN and IR, whereas the prevalence of AN in non-IR patients was negligible. Insulin has also been shown to stimulate androgen production by stimulating ovarian theca and stromal cells directly as well as by interaction through ovarian insulin growth factor-I (IGF-I) receptors.[3,23] Clinically, hyperandrogenemia often manifests itself by hirsutism, as was the case in the current study in which a significant association was seen between hirsutism and IR. Interestingly, this association was demonstrated despite significantly lower circulating testosterone levels in IR compared with non-IR patients. Higher circulating insulin levels in IR patients may potentially lower SHBG levels leading to higher circulating free testosterone levels, which may explain the greater prevalence of hirsutism in those patients compared with non-IR ones. We observed significantly lower mean levels of LH and LH/FSH ratios in IR patients, which may explain lower circulating testosterone levels in those patients compared with non-IR patients. In fact, the observation of low LH in hyperinsulinemic patients is consistent with prior reports suggesting 2 possible distinct phenotypes of PCOS, a low-LH and high-insulin group and a high-LH and low-insulin group.[24] Such observations again support the need to subclassify PCOS patients into IR and non-IR groups.

IR and compensatory hyperinsulinemia can inhibit follicular development and, subsequently, ovulation by leading to a hyperandrogenic intraovarian microenvironment,[25,26] and by altering gonadotropin secretion.[27,28] It has been previously demonstrated that among women with PCOS, those women with severe IR are more likely to fail to respond to ovulation induction with CC than women lacking IR.[29] Similarly, in the current study, PCOS women with IR were more than 5 times as likely to be resistant to CC as women without IR, despite no significant difference in mean BMI or mean CC dose used between the 2 groups. These findings again suggest that IR should be designated as a unique state in PCOS and point to the need to individualize treatment in such patients, perhaps through use of insulin-sensitizing medications such as metformin. To date, no controlled trials have been performed to compare ovulation induction in response to insulin sensitizers between IR and non-IR PCOS patients.

The insulin receptor consists of 2 α-subunits located extracellularly and containing the ligand-binding domain, and 2 β-subunits spanning the cell membrane and containing intracellular protein tyrosine kinase activity.[30,31] It has been suggested that IR in PCOS results from a postbinding defect in insulin action. Dunaif et al[14] demonstrated no change in insulin binding or receptor affinity in cultured fibroblasts and adipocytes, whereas decreased insulin-dependent receptor tyrosine phosphorylation was demonstrated in cells isolated from 50% of PCOS patients. In the current study, we were able to demonstrate reduced APIR by using a simple technique that allowed separate enzymatic quantification of total and autophosphorylated erythrocyte insulin receptor. As expected, mean APIR/TIR ratios increased with increasing insulin concentrations, consistent with increased autophosphorylation of the receptor with more bound insulin, in women with no PCOS, as well as PCOS women with and without IR. Insulin resistant PCOS women were found to have significantly lower APIR/TIR ratios than non-IR PCOS women and non-PCOS controls, particularly at higher insulin concentrations.

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suggesting reduced capacity for APIR in such women. Similar APIR/TIR ratios were observed between women with no PCOS and PCOS women lacking IR, suggesting that the autophosphorylation receptor defect is not a universal finding in PCOS, again supporting the need to subclassify PCOS patients as either IR or non-IR. A correlation was found between fasting G/I ratios and APIR/TIR ratios in IR patients, substantiating a concordance between clinical and cellular measures of IR. Finally, although IR had previously been demonstrated in both lean and obese PCOS women,[19,20] the effect of obesity on the cellular insulin receptor defect was clearly demonstrated with a significant inverse correlation between BMI and APIR/TIR ratios, in the current study, contributing to the available body of evidence showing exacerbation of IR with obesity.

In conclusion, IR in PCOS is not a universal finding. Clinically, IR in PCOS is associated with obesity, AN, hirsutism, and resistance to ovulation induction with CC. Moreover, women with PCOS who have IR are likely to exhibit lower serum LH, LH/FSH ratios, and testosterone levels than non-IR patients. Therefore, patients with IR represent a unique subphenotype of PCOS with clinical and biochemical characteristics different from patients lacking IR. Finally, IR patients appear to have defective APIR, a key element in insulin action, and a possible mechanism for IR in PCOS.

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