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Decreased Insulin Receptor (IR) Autophosphorylation in Fibroblasts from Patients with PCOS: Effects of Serine Kinase Inhibitors and IR Activators

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Insulin resistance is characteristic of many patients with polycystic ovary syndrome (PCOS). Several studies have suggested that a decrease in insulin receptor (IR) autophosphorylation is a significant component of this resistance. In this study, we have used a highly sensitive ELISA to measure IR tyrosine phosphorylation in fibroblasts from patients with PCOS and healthy control women. After the stimulation of intact fibroblasts with insulin, IR tyrosine phosphorylation in cells from the PCOS patients was decreased by approximately 40% when compared with controls. However, when IR were first immunocaptured from fibroblasts and then stimulated with insulin, neither basal nor insulin-stimulated IR autophosphorylation was different between the two groups, suggesting that a factor independent of the IR was involved. To examine the role of increased serine kinase activity in decreased IR autophosphorylation in PCOS, fibroblasts from PCOS patients were pretreated with inhibitors of serine kinases before insulin stimulation. Pretreatment with H7, a nonspecific protein kinase inhibitor, completely reversed the decrease in insulin-stimulated IR autophosphorylation. Pretreatment with H89, an inhibitor of protein kinase A, partially reversed this function, whereas pretreatment with Gö6983, an inhibitor of protein kinase C, was without effect. We next studied the effects of two small molecule activators of the IR tyrosine kinase: TLK16998 and Merck L7. Both TLK16998 and Merck L7 were able to reverse the impaired insulin-stimulated IR autophosphorylation. In summary, a factor(s) extrinsic to the IR cause impaired IR signaling in fibroblasts from patients with PCOS. Reversal of the impaired IR signaling by inhibitors of serine kinase activity suggests that serine kinase-mediated pathways may be involved in the insulin resistance. Moreover, the observation that TLK16998 and Merck L7 improved IR tyrosine phosphorylation in fibroblasts from patients with PCOS suggests that specific pharmacological therapies might be developed to treat the insulin resistance in PCOS. (*J Clin Endocrinol Metab* 87: 4088–4093, 2002)

POLYCYSTIC OVARY SYNDROME (PCOS) is a common and complex endocrine disorder with unknown etiology (1–3). PCOS is characterized by hyperandrogenism, chronic anovulation, and, frequently, profound insulin resistance (3, 4). Insulin resistance in PCOS is secondary to a postbinding defect in insulin receptor (IR) signaling (5–7). The cellular response to insulin is mediated through the IR, which is a tetrameric protein consisting of two identical extracellular α-subunits that bind to insulin and two identical transmembrane β-subunits that have intracellular tyrosine kinase activity (8–10). When insulin binds to the α-subunit of the receptor, the β-subunit tyrosine kinase undergoes autophosphorylation, resulting in the activation of the IR tyrosine kinase activity (11, 12). Once activated, the IR tyrosine kinase phosphorylates a number of intracellular targets, including the IR substrate (IRS) family of proteins, triggering a cascade of events ultimately leading to increased glucose use (13–15).

Dunaif *et al*. (16) reported that IR autophosphorylation was decreased in fibroblasts in approximately 50% of patients with PCOS (16). These investigators attributed this diminished IR function to increased serine phosphorylation of the IR by an unknown serine kinase activity. Increased serine phosphorylation of the IR or IRS decreases the extent of tyrosine phosphorylation and leads to impaired insulin action (11, 17, 18). Because these effects were observed in cultured fibroblasts, the data suggested that these abnormalities in IR function were intrinsic to the cell and not due to the metabolic state of the patients. Moreover, when the IRs were purified from associated proteins, IR autophosphorylation was normal, and serine kinase activity was not detected. These observations suggested that the serine kinase involved was not a component of the IR (19).

The study by Dunaif *et al*. (16), therefore, strongly implicated that decreased IR function and excess serine kinase activity were key features of PCOS. These findings suggested that inhibition of serine kinase might reverse the inhibition of the IR autophosphorylation and provide information as to the nature of the kinase involved. Recently, small molecule, nonpeptide, IR activators have been developed that restore IR autophosphorylation in insulin-resistant cells, but their effects on PCOS fibroblasts have not yet been studied.

We have recently developed and validated a highly specific and sensitive ELISA to measure IR tyrosine phosphorylation in fibroblasts and other cells (20). In the present study, we have used this ELISA to investigate IR function in fibroblasts from patients with PCOS and control individuals, and

Abbreviations: IR, Insulin receptor; IRS, IR substrate; PCOS, polycystic ovary syndrome; PKA, protein kinase A; PKC, protein kinase C.

we studied the effects of inhibitors of serine kinases on IR autophosphorylation. In addition, we studied the effects of two new IR activators, Merck L7 and TLK16998 (21–24). In these studies, we find diminished IR autophosphorylation in PCOS fibroblasts. This effect is blocked by inhibiting cellular serine kinase activity and is reversed by the IR activators Merck L7 and TLK16998.

Subjects and Methods

Subjects

Skin fibroblast cell lines were established in seven women with PCOS and four control women aged 18–43 yr as part of previously reported studies (14, 16) at the Mt. Sinai School of Medicine (New York, NY) and at the Pennsylvania State University College of Medicine (Hershey, PA). The Institutional Review Boards of both institutions approved the studies, and all women gave written informed consent. The women were in good health and, for at least 1 month (and 3 months for oral contraceptives) before study, were off medications known to affect sex hormone, lipid, or carbohydrate metabolism. The diagnosis of PCOS was made by the presence of chronic anovulation (six or fewer menses per year) in association with elevated circulating levels of testosterone, androstenedione, and/or free and weakly bound (unbound) testosterone, and exclusion of other causes of hyperandrogenism (16). The PCOS subjects were subjects 2, 5, 6, 8, 12, 13, and 15 in our previous study of IR phosphorylation in cultured skin fibroblasts (16). Three of these subjects had impaired glucose tolerance. The control women had menses every 27–32 d, no hirsutism, and no personal history of or first-degree relative with diabetes mellitus. Their circulating androgen levels were within the normal range established for reproductively normal premenopausal women in the follicular phase of the menstrual cycle (16). The control women had participated in our previous studies of insulin action in cultured skin fibroblasts (14, 16). The clinical, reproductive, and metabolic features of the PCOS and control women have been previously reported (14, 16).

Chemicals

TLK16998 and Merck L7 were kindly provided by Telik, Inc. (South San Francisco, CA), and Merck & Co., Inc. (Rahway, NJ), respectively. H89 (*N*-(2-[p-bromocinnamylamino]-ethyl)-5-isoquinolinesulonamide) was purchased from Sigma (St. Louis, MO). H7 [1-(5-isoquinolinesulfonyl)-2-methylpipelazine, 2HCL] and Gö6983 were purchased from Calbiochem (San Diego, CA). Tetramethylbenzidine (TMB) reagent kit was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Skin biopsies and fibroblast cultures

Skin fibroblast cultures were established from forearm skin punch biopsies as previously described (16). The outgrown fibroblasts from the primary biopsies of control individuals and patients with PCOS were subcultured in DMEM containing 4.5 g/liter D-glucose supplemented with 10% (vol/vol) fetal calf serum. Penicillin (10 U/ml), fungizone (0.25 μg/ml), and streptomycin (10 μg/ml) were routinely added to cultures. Cells were cultivated at 37 C in a 5% CO_2-enriched, humidified atmosphere. Cells were used for study between the sixth and ninth passage.

ELISA for intact-cell IR autophosphorylation

Fibroblasts were grown in six-well plates until confluent and then serum starved [0.1% (wt/vol) BSA] for 15 h before insulin stimulation (10 min at 37 C). After treatment, cells were washed extensively in ice-cold PBS, scraped from culture plates, and incubated for 60 min at 4 C in lysis buffer [50 mM HEPES, pH 7.6, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonylfluoride, and 2 mM sodium orthovanadate]. Lysates were centrifuged (15,000 $\times$ *g* for 30 min at 4 C) to remove insoluble materials. The protein content in each sample was measured using Bio-Rad protein assay dye reagent concentrate according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of cell lysates (50 μg protein) were applied to a 96-well microtiter plate previously coated with an antihuman IR monoclonal antibody, MA-20 (25). After 18 h incubation at 4 C, microtiter plates were washed with 20 mM Tris (pH 7.4), 150 mM NaCl, and 0.05% (vol/vol) Tween 20 (TBST). Subsequently, biotin-conjugated anti-phosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) was added and followed by streptavidin-horseradish peroxidase (Pierce Chemical Co., Rockford, IL). TMB (3,3′,5,5′-tetramethylbenzidine) was used as the chromogenic substrate for horseradish peroxidase. The autophosphorylation signal was detected using Microplate Reader II-Multiskan Mcc/340 (DuPont, Boston, MA) by reading absorption at 450 nm. In some experiments, fibroblasts were pretreated with serine kinase inhibitors, IR tyrosine kinase activators, or vehicle (as a control) as indicated in figure legends before stimulation with insulin. Stock solutions (20 mM in dimethylsulfoxide) of test compounds were prepared fresh before each experiment. To improve compound stability, Merck L7 was diluted to the desired concentration in cell medium containing ascorbic acid (final concentration, 200 μg/ml; also included in vehicle-treated cells). Control cells were incubated with the identical concentration of vehicle (0.1% final dimethylsulfoxide).

ELISA for IR autophosphorylation in immunocaptured receptors

Fibroblasts were cultured to confluence, serum starved for 15 h, solubilized and proteins quantified as described above. Lysates of fibroblasts containing 50 μg protein were applied to 96-well microtiter plates coated with monoclonal antihuman IR antibody, MA-20. Insulin (0–100 nM) was added to the immunocaptured IR along with 10 μM of ATP for 1 h in 96-well microtiter plates. Plates were washed in TBST and processed for IR tyrosine autophosphorylation as described above.

Statistics

Data are expressed as means $\pm$ SEM. Differences between means were assessed by the *t* test or one-way ANOVA using GraphPad Prism version 3.02 for Windows (GraphPad Software, Inc., San Diego, CA; www.graphpad.com). *Post hoc* comparisons were performed using Dunnett's test or the Newman-Keuls test for multiple comparisons. Statistical significance was accepted at *P* value less than 0.05.

Results

Insulin-stimulated IR β-subunit autophosphorylation of fibroblasts from PCOS vs. controls

Intact fibroblasts from control and PCOS patients were treated with increasing concentrations of insulin up to 100 nM, and autophosphorylation of the IR β-subunit was measured (Fig. 1). In fibroblasts from normal individuals, an effect of insulin was detectable at 0.3 nM, the one half maximal effect at 3 nM, and the maximal effect at 10 nM. When compared with controls, fibroblasts from PCOS patients had an approximate 40% reduced response to insulin at all concentrations tested ($P < 0.05$ for insulin concentrations $\geq$1 nM). The slight difference in basal IR autophosphorylation was not statistically significant.

Next, IRs were immunocaptured, washed free from cellular components, and then stimulated with insulin. Basal and insulin-stimulated IR autophosphorylation was not significantly different between the patients with PCOS and the controls (Fig. 2). These data confirm earlier data obtained in fibroblasts (16) and suggest that a factor(s) extrinsic to the IR is responsible for the decreased autophosphorylation of PCOS.

Serine kinase-mediated regulation of IR β-subunit tyrosine kinase activity

A previous study indicated that this factor could be a serine kinase activity (16). Therefore, we investigated

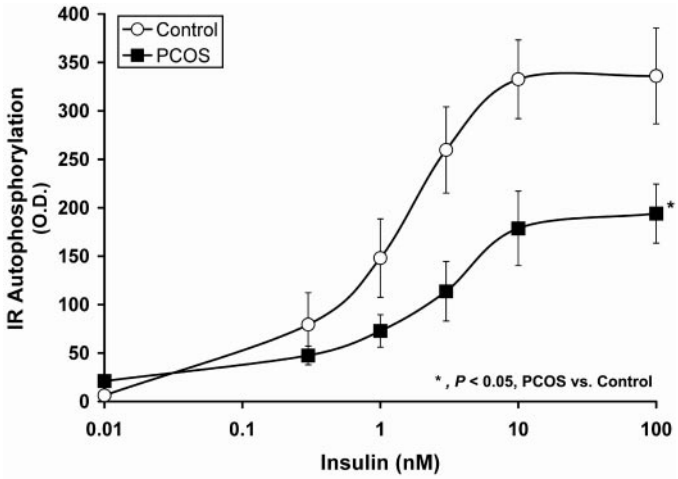

FIG. 1. Autophosphorylation of IRs in fibroblasts from control subjects and patients with PCOS. Fibroblasts were incubated with increasing concentrations of insulin for 10 min at 37 C, and lysates were prepared. An equal amount of protein from each preparation was analyzed. Autophosphorylation of the IR β-subunit was performed using an ELISA as described in *Subjects and Methods*. Data points represent means ± SEM for separate experiments with fibroblasts from four control subjects and seven patients with PCOS. *, $P < 0.05$, PCOS *vs.* control (*t* test, paired).

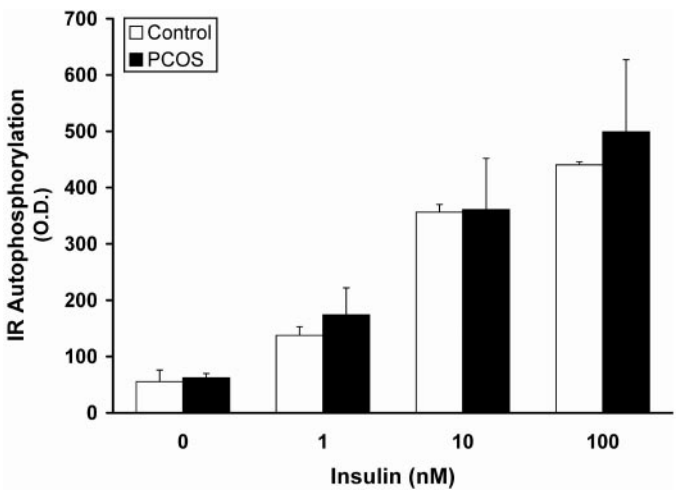

FIG. 2. Autophosphorylation of immunopurified IRs from fibroblasts from control subjects and patients with PCOS. Cell lysis, protein determination, immunopurification of IR β-subunit, and ELISA for autophosphorylation were performed as described in *Subjects and Methods*. Immunopurified IRs were incubated with increasing concentrations of insulin. Data represent means ± range for separate experiments with fibroblasts from two control subjects and two patients with PCOS. Not significant, PCOS *vs.* control (*t* test, paired).

whether chemical inhibitors of serine kinase activity could affect IR autophosphorylation in fibroblasts from patients with PCOS. Preliminary experiments using specific enzyme assays were performed to identify the maximally effective concentration of each inhibitor (data not shown). H7 (2 μM), an inhibitor of cyclic nucleotide-dependent protein kinase and protein kinase C (PKC) (26), completely reversed the impaired IR autophosphorylation (Fig. 3). Partial reversal was observed with H89, a selective inhibitor of protein kinase A (PKA; Ref. 27). Gö6983, a selective inhibitor of PKC, at

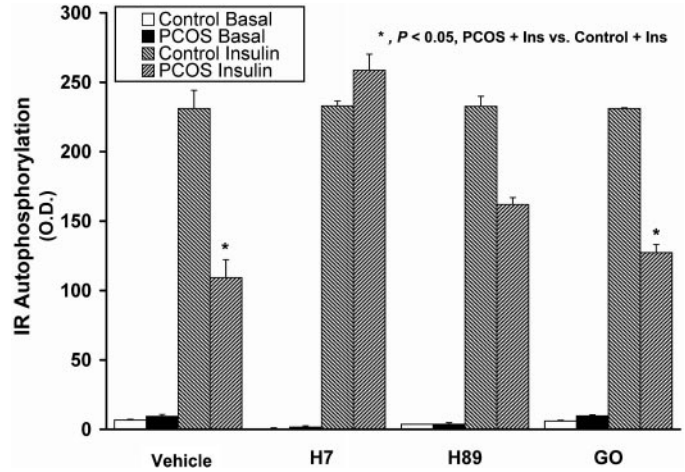

FIG. 3. Effects of serine kinase inhibitors on IR autophosphorylation in fibroblasts from control subjects and patients with PCOS. Fibroblasts were pretreated for 20 min in the absence (vehicle control) or presence of 2 μM H7, an inhibitor of cyclic nucleotide-dependent protein kinase; 20 μM H89, an inhibitor of PKA; and 100 nM Gö6983, an inhibitor of PKC. Where indicated, fibroblasts were stimulated with insulin (100 nM). Cell lysis, protein determination, and ELISA for autophosphorylation were performed as described in *Subjects and Methods*. Data represent means ± SEM for separate experiments with fibroblasts from three control subjects and three patients with PCOS. *, $P < 0.05$, compared with control subjects treated with insulin (ANOVA and Newman-Keuls *post hoc* test). In fibroblasts pretreated with H7 or H89 followed by insulin, there was no significant difference between PCOS compared with control (ANOVA).

concentration (100 nM) sufficient to block PKC isoforms α, β, γ, δ, and ζ (28, 29), did not significantly modify IR tyrosine phosphorylation of fibroblasts from the PCOS (Fig. 3). In fibroblasts from the control individuals, H7, H89, and Gö6983 did not affect insulin-stimulated IR autophosphorylation (Fig. 3).

Modification of IR β-subunit autophosphorylation by small molecule IR activators

Small, nonpeptide molecules have been shown to activate IR β-subunit tyrosine kinase activity (21–24). Accordingly, in fibroblasts from normal subjects, both TLK16998 and Merck L7 increased IR tyrosine kinase activity ($P < 0.05$; Fig. 4A). In addition, Merck L7 significantly increased basal autophosphorylation ($P < 0.05$). In fibroblasts from patients with PCOS, TLK16998 significantly enhanced insulin-stimulated IR β-subunit autophosphorylation at both submaximal and maximal concentrations of insulin. Merck L7 enhanced insulin-stimulated IR β-subunit autophosphorylation at submaximal concentrations (1 and 3 nM; $P < 0.05$), but not at a maximal concentration of insulin (Fig. 4B).

Discussion

The present study was performed to determine whether decreased IR autophosphorylation in fibroblasts from PCOS patients could be reversed by either inhibition of serine kinase activity or IR activation. In intact fibroblasts, using a sensitive and specific ELISA technique, we found a 40% decrease in this function. The decrease in IR autophosphorylation was prevented in the presence of inhibitors of serine

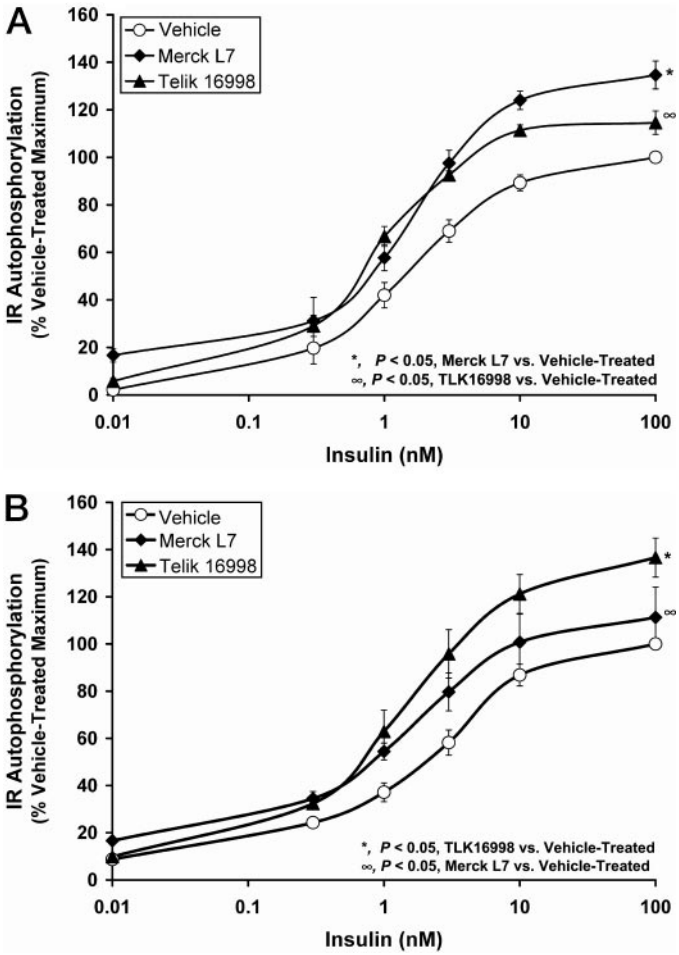

FIG. 4. Effects of IR activators on IR autophosphorylation in fibroblasts from control subjects and patients with PCOS. Fibroblasts from control subjects (A) and patients with PCOS (B) were incubated in the absence (vehicle) or presence of Merck L7 (20 μM) or TLK16998 (20 μM) for 10 min, and then stimulated with increasing concentrations of insulin for 10 min at 37 C. Lysates were prepared, and an equal amount of protein from each preparation was analyzed. Autophosphorylation of the IR β-subunit was performed using an ELISA as described in *Subjects and Methods*. In each figure, absorbance at 450 nm for the vehicle-treated fibroblasts stimulated with 100 nM insulin was set to 100%, and the other treatment conditions expressed as percentage vehicle-treated maximum. Data points represent means ± SEM for separate experiments with fibroblasts from four control subjects and four patients with PCOS. A (Control subjects), $P < 0.05$, Merck L7 and TLK16998 *vs.* vehicle (repeated measures ANOVA and Dunnett's *post hoc* test). In addition, in the absence of insulin (basal), $P < 0.05$, Merck L7 *vs.* vehicle (ANOVA and Dunnett's *post hoc* test). B, (Patients with PCOS), $P < 0.05$, Merck L7 and TLK16998 *vs.* vehicle (repeated measures ANOVA and Dunnett's *post hoc* test). In addition, $P < 0.05$, Merck L7 *vs.* vehicle for 1 and 3 nM insulin, and TLK16998 *vs.* vehicle for insulin concentrations ≥1 nM (ANOVA and Newman-Keuls *post hoc* test).

kinase activity. In contrast, in isolated purified IR from fibroblasts, no difference in IR autophosphorylation could be detected. These studies support the original observations of Dunaif *et al.* (16) that decreased IR tyrosine kinase activity and increased serine kinase activity occur in fibroblasts from patients with PCOS.

Although the present study suggests a role for decreased IR autophosphorylation in the insulin resistance of PCOS, it is likely that other defects might be present. Book and Dunaif (30) have found additional evidence that, in fibroblasts from patients with PCOS, there is a greater defect in metabolic *vs.* mitogenic signaling, suggesting additional defects in downstream signaling. Ciaraldi *et al.* (6), using adipocytes from patients with PCOS, reported a 30% decrease in IR autophosphorylation, but an 8-fold decrease in insulin-stimulated glucose transport. They concluded, therefore, that a major postreceptor defect in IR signaling was also present.

Increased serine phosphorylation of the IR or downstream signaling components such as IRS decreases the extent of their tyrosine phosphorylation and leads to impaired insulin action (31–37). For instance, serine phosphorylation of the IR via a number of mechanisms impairs its ability to undergo autophosphorylation. Moreover, serine phosphorylated forms of IRS molecules are less able to associate with the IR and downstream target molecules, especially phosphatidylinositol 3-kinase (31, 38), resulting in impaired insulin action, including protein kinase B activation, and glucose transport. Recently, several serine kinases have been implicated in insulin resistance, including a certain isoform of PKC (35) and the IκB kinase (37).

We observed that H7, a nonselective protein kinase inhibitor, and H89, a relatively selective inhibitor of PKA, reversed or partially reversed, respectively, the impaired IR autophosphorylation of fibroblasts from the PCOS. In contrast, a relatively selective PKC inhibitor, Gö6983, at a concentration sufficient to block PKC isoforms α, β, γ, δ, and ζ, did not significantly modify the insulin response in insulin-resistant PCOS fibroblasts. These data suggest that a serine kinase, perhaps associated with a PKA-regulated pathway, might be involved in the insulin resistance. This pathway, which remains to be defined, could potentially account for both receptor and postreceptor defects observed in cells from patients with PCOS.

Small-molecule activators of IR might represent a new class of antidiabetic agents (39, 40). In the present study, we found that TLK16998 increased IR autophosphorylation in fibroblasts from both control subjects and insulin-resistant patients. This increase in IR autophosphorylation is in agreement with recent studies (41), in which TLK16998 enhanced insulin-stimulated IR autophosphorylation in insulin-resistant cells. Merck L7 also increased IR autophosphorylation in control subjects. In fibroblasts from patients with PCOS, Merck L7 enhanced insulin-stimulated IR β-subunit autophosphorylation at submaximal concentrations of insulin, but not at maximal insulin. In contrast, TLK16998 stimulated IR β-subunit autophosphorylation at both submaximal and maximal concentrations of insulin.

TLK16998 and Merck L7 are chemically distinct compounds. TLK16998 has a polysulfonic acid moiety (24), whereas Merck L7 is a quinone-like compound (21). In a previous study, both compounds improved insulin sensitivity in cells that either overexpressed membrane glycoprotein PC-1 or were incubated with TNF-α. However, only TLK16998 overcame insulin resistance in cells incubated with phorbol esters, activators of PKC (41). The present finding that both TLK16998 and Merck L7 were effective, to a varying degree, in fibroblasts from patients with PCOS is in agreement with the earlier observation (41). Insulin-sensi-

tizing agents such as metformin, troglitazone, rosiglitazone, and D-chiroinositol produce significant improvements in the reproductive abnormalities of PCOS (42–49). Specific serine kinase inhibitors or IR tyrosine kinase activators might also hold promise as therapeutic agents for not only the metabolic but also the reproductive abnormalities associated with PCOS.

In addition to the ability of serine phosphorylation to reduce IR activity (16), previous work has demonstrated that increased serine phosphorylation of cytochrome P450c17, a microsomal enzyme normally expressed in ovaries and adrenal tissue, increases its 17,20-lyase activity (50). An increase in the activity of this enzyme would promote the increased androgen production characteristic of PCOS (51). These observations have prompted the idea that an increase in a serine kinase activity targeting both the IR and P450c17 could serve as a mechanistic link between the insulin resistance and hyperandrogenism observed in PCOS (52). To explore this possibility, P450c17 was stably expressed in fibroblasts from normal individuals and patients with PCOS (a hyperphosphorylating environment), and the activity of 17,20-lyase was measured (53). This study found no correlation between 17,20-lyase activity and the clinical phenotype of the donors of cells. However, it is quite possible that the hyperphosphorylation and activation of P450c17 is mediated by the tissue-specific expression of the relevant kinase or, alternatively, by the need for accessory proteins not expressed in fibroblasts. Thus, the unifying hypothesis of the enhanced activity of a single serine kinase acting on the IR and P450c17, resulting in insulin resistance and hyperandrogenism, remains appealing but requires experimental validation.

In summary, the data support the concept that decreased IR tyrosine kinase activity is a feature of the insulin resistance found in patients with PCOS. In addition, serine kinases may play a role in this type of insulin resistance. In tissues from insulin-resistant women with gestational diabetes, enhanced serine kinase activity was shown to play a role in decreased IR autophosphorylation (54). Taken together, these data along with recent data from Griffin *et al.* (35) and Yuan *et al.* (37) implicating serine kinase activity in the insulin resistance of obesity suggest that further investigations into the family of serine kinases and the use of IR tyrosine kinase activators may lead to improved treatments for the insulin resistance in patients with PCOS.

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