Excessive Insulin Receptor Serine Phosphorylation in Cultured Fibroblasts and in Skeletal Muscle

A Potential Mechanism for Insulin Resistance in the Polycystic Ovary Syndrome

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Abstract

We investigated the cellular mechanisms of the unique disorder of insulin action found in the polycystic ovary syndrome (PCOS). Approximately 50% of PCOS women (PCOS-Ser) had a significant increase in insulin-independent β-subunit [^{32}P]phosphate incorporation (3.7-fold, $P < 0.05$ vs other groups) in skin fibroblast insulin receptors that was present in serine residues while insulin-induced tyrosine phosphorylation was decreased (both $P < 0.05$ vs other groups). PCOS skeletal muscle insulin receptors had the same abnormal phosphorylation pattern. The remaining PCOS women (PCOS-n1) had basal and insulin-stimulated receptor autophosphorylation similar to control. Phosphorylation of the artificial substrate poly GLU4:TYR1 by the PCOS-Ser insulin receptors was significantly decreased ($P < 0.05$) compared to control and PCOS-n1 receptors. The factor responsible for excessive serine phosphorylation appeared to be extrinsic to the receptor since no insulin receptor gene mutations were identified, immunoprecipitation before autophosphorylation corrected the phosphorylation defect and control insulin receptors mixed with lectin eluates from affected PCOS fibroblasts displayed increased serine phosphorylation. Our findings suggest that increased insulin receptor serine phosphorylation decreases its protein tyrosine kinase activity and is one mechanism for the post-binding defect in insulin action characteristic of PCOS. (*J. Clin. Invest.* 1995. 96:801–810.) Key words: insulin receptors • signal transduction • phosphoserine • polycystic ovary syndrome • insulin resistance

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1. *Abbreviations used in this paper:* DGGE, denaturing gradient gel electrophoresis; hIR, human insulin receptor; NIDDM, non-insulin dependent diabetes mellitus; PCOS, polycystic ovary syndrome; PCOS-n1, PCOS insulin receptor β-subunits with phosphotyrosine and phosphoserine content similar to control; PCOS-Ser, PCOS insulin receptor β-subunits with increased phosphoserine, decreased phosphotyrosine content.

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Introduction

Polycystic ovary syndrome (PCOS)[1] is probably the most common endocrine disorder of premenopausal women (1, 2). This is a condition of unknown etiology characterized by hyperandrogenism, chronic anovulation and, frequently, substantial insulin resistance (1, 3, 4). Obesity and PCOS have a synergistic negative impact on insulin action such that 20% of obese PCOS women have impaired glucose intolerance or non-insulin dependent diabetes mellitus (NIDDM) by their third decade (3, 4). It can be extrapolated from these data that PCOS-related insulin resistance is an important cause of glucose intolerance in premenopausal women (5). The cellular mechanisms of insulin resistance in PCOS, however, appear to differ from those in the other common insulin resistant states of obesity and NIDDM (6). There is a striking shift to the right in the insulin dose–response curve for glucose uptake in adipocytes, without changes in adipocyte insulin binding, greater than that seen with obesity alone (6, 7). This defect is, in part, explained by a decrease in insulin-stimulated insulin receptor autophosphorylation (7). There is also a more modest but significant decrease in maximal rates of insulin-mediated glucose uptake in adipocytes in PCOS (6). This is secondary to decreased abundance of the insulin-regulatable GLUT4 glucose transporter (8). Although caution must be exercised in extrapolating cellular mechanisms from in vivo dose–response curves, such studies suggest that similar defects are present in muscle, the major insulin target tissue (6).

In NIDDM and in obesity many abnormalities in insulin action are secondary to hyperinsulinemia and hyperglycemia (9–13). In contrast, in PCOS such defects are independent of glucose tolerance, insulin levels, and body fat topography (6, 8). Some investigators have suggested that hyperandrogenism decreases insulin action (14, 15). In our studies, circulating androgen levels have not been correlated with parameters of insulin sensitivity and we have found that suppression of hyperandrogenism achieved through "medical castration" with a long-acting GnRH analog does not alter insulin action in PCOS (3, 4, 6, 16). However, other studies (17, 18) have suggested that androgens do contribute to insulin resistance in PCOS. Thus, it remains possible that defects in insulin action in PCOS represent acquired rather than intrinsic abnormalities. To investigate this possibility and to further assess the mechanisms of the post-binding defect in insulin receptor-mediated signal transduction, we examined insulin binding and receptor protein tyrosine kinase activity in cultured PCOS skin fibroblasts that had been removed from the in vivo environment for generations as well as in skeletal muscle. We have identified a novel abnormality of insulin receptor phosphorylation characterized by increased insulin-independent phosphate incorporation into serine

residues, decreased insulin-dependent tyrosine autophosphorylation and decreased artificial substrate phosphorylation in ~ 50% of PCOS women. Serine phosphorylation of the insulin receptor is postulated to be a mechanism for terminating insulin signaling (19–22). PCOS is thus the first insulin resistant state in which this mechanism appears to be operative.

Methods

Materials. DME, FBS, penicillin-streptomycin solution, and EDTA were obtained from Fisher Scientific (Pittsburgh, PA) Phosphate-free DME was obtained from GIBCO BRL (Gaithersburg, MD) Hepes, Triton X-100 and DTT were obtained from Boehringer Mannheim (Indianapolis, IN) Wheat germ agglutinin (WGA) was obtained from Calbiochem (San Diego, CA). Protein A Sepharose CL-4B was purchased from Pharmacia LKB (Piscataway, NJ) Centricon-30 concentrators were purchased from Amicon Inc. (Beverly, MA), and *N*-ethylmorpholine was purchased from Pierce (Rockford, IL). Human insulin was purchased from E. Lilly Co. (Indianapolis, IN) and IGF-I was purchased from Bachman (Torrance, CA). A 14-tyrosine [^{125}I]insulin and [^{32}P]orthophosphate were purchased from Dupont (Wilmington, DE). The antiphosphotyrosine antibody, PY20, and [^{32}P]-ATP were purchased from ICN Biochemicals (Costa Mesa, CA). The polyclonal anti-insulin receptor antibody was purchased from Transduction Laboratories (Lexington, KY). The monoclonal anti-insulin receptor antibody, 18–44, was the gift of Dr. Kenneth Siddle, and the monoclonal anti–IGF-I receptor antibody, αIR3, was the gift of Dr. Jeffrey Pessin. NIH 3T3 fibroblasts that overexpress transfected human insulin receptor cDNA were the gift of Dr. J. Whittaker (23). The remaining reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Subjects. 16 women with PCOS (10 obese, 6 nonobese) and 9 control women (5 obese, 4 nonobese) matched for age and weight were studied. 14 subjects, PCOS (PCOS Subjects 1–8 and 11–14, Tables I and II) and control (4 obese and 4 nonobese), had participated in our previous studies of insulin action on glucose transport examined in isolated adipocytes (6, 8). Two additional PCOS women and one control woman had muscle biopsies; these PCOS women had insulin resistance documented by euglycemic glucose clamp studies (6). Subjects were between the ages of 21–43 yr, in good health, euthyroid, and none had taken any medications known to affect gonadal function or carbohydrate metabolism for at least three months prior to study. The diagnosis of PCOS was made when testosterone, non-sex hormone binding globulin bound testosterone and/or androstenedione levels were elevated greater than two standard deviations above the female control mean in association with chronic anovulation, as evidenced by either amenorrhea or oligomenorrhea with six or fewer menses per year. No PCOS woman had late-onset 21-hydroxylase deficiency by a 1 h ACTH stimulation test or hyperprolactinemia (1). Most of the PCOS women also had polycystic ovaries on ovarian ultrasound (2). Hirsutism was not used as a selection criterion because hyperandrogenism can be present without any clinical expression due to differences among individuals in target tissue sensitivity to androgens (24). Acanthosis nigricans was not used to stratify subjects because we have shown that the skin lesion is an epiphenomenon of insulin resistance rather than a specific marker for hyperandrogenism (25).

Normal nondiabetic control women had regular menses every 27–32 d; no hirsutism or hyperandrogenism were present. There was no history of NIDDM or insulin-dependent diabetes mellitus in the first degree relatives of the control women. Obesity was defined as a body mass index (BMI) > 27 kg/m^2 while nonobese women had a BMI < 27 kg/m^2. Studies were approved by the institutional review boards of the Mt. Sinai School of Medicine and the Pennsylvania State University College of Medicine. Written informed consent was obtained on all subjects before study.

A 75 gram oral glucose tolerance test was performed in the morning after a 3 d 300 gram carbohydrate preparatory diet and glucose and insulin levels were obtained every 30 min for 2 h. All of the control women had normal glucose tolerance whereas some of the PCOS women had impaired glucose tolerance or diabetes mellitus by WHO criteria (5). None of the PCOS women had fasting hyperglycemia. The clinical features and glucose tolerance data are summarized in Table I and the adipocyte insulin action data in Table II. Most of these data have been reported previously (6, 8) and are reported again in this study to describe the phenotype of the PCOS women.

Table I. Clinical Features and Glucose Tolerance

Subject	Racial/ ethnic	Age (yr)	BMI (kg/m^2)	Glucose (mM) 0 min	Glucose (mM) 120 min	Insulin (pM) 0 min	Insulin (pM) 120 min
Obese PCOS							
1	C	40	48.9	5.4	8.5	101	761
2	H	40	41.7	6.2	10.5	804	5442
3	C	34	39.7	5.0	9.9	237	1099
4	H	24	30.2	4.7	7.7	187	1292
5	H	32	55.2	4.5	5.8	172	610
6	C	43	36.1	5.1	9.1	287	1587
7	C	21	31.2	4.5	6.8	165	783
8	B	28	27.9	5.2	8.3	86	495
9	C	21	45.1	4.8	7.3	244	1630
10	C	26	46.9	5.8	8.3	165	1106
Mean±SEM		33±3	38.6±3.5	5.1±.2	8.3±.6	255±82	1509±577
Obese control							
n = 5							
mean±SEM		35±2	32.8±2.5	5.1±.2	6.2±.5	108±41	316±79
Nonobese PCOS							
11	C	37	23.0	5.0	5.4	72	323
12	C	26	26.3	5.1	5.2	22	129
13	C	23	22.8	4.3	4.9	29	280
14	C	28	26.4	5.0	8.3	101	984
15	H	23	25.4	5.0	7.6	144	819
16	C	42	20.6	5.2	6.8	43	187
Mean±SEM		30±3	24.1±.9	4.9±.1	6.4±.6	68±19	454±146
Nonobese control							
n = 4							
mean±SEM		25±3	22.4±1.2	4.6±.2	5.3±.5	50±9	355±127

C, Caucasian; *H*, Hispanic; *B*, Black.

Skin biopsies and fibroblast cultures. After local anesthesia with 1% lidocaine with epinephrine, a 2-mm punch biopsy was performed under sterile conditions on the volar aspect of the forearm. The skin was cultured in DME (4 grams/liter glucose) supplemented with 10% FBS and 1% penicillin/streptomycin solution in a humid 37°C incubator with 5% CO_2 (26). In 7–14 d, fibroblast outgrowth from the primary biopsy was evident; growth continued to near confluence before further subculturing was performed. Cells were used for study between the fourth and eighth passage.

Muscle biopsies. Muscle biopsies were performed in the morning after an overnight fast and a 3 d 300 gram carbohydrate preparatory diet in two PCOS women and one control woman. After local anesthesia with 1% lidocaine, a 5-cm incision was made in the left axilla under sterile conditions and ~ 1 gram of pectoralis major muscle was removed. Approximately 1 gram of rectus abdominus muscle was taken at the time of indicated surgery in an additional normal woman. Muscle was immediately frozen in liquid nitrogen until use.

Insulin binding assay. Fibroblasts were grown to confluence in 6-well tissue culture dishes in supplemented DME prior to incubation in serum-free medium containing 0.1% BSA for 48 h. Previous studies had shown that this period of serum-starvation was necessary to achieve maximal insulin binding (26). The binding assay was performed at room temperature for 4 h with radiolabeled insulin and unlabeled insulin in buffer containing 50 mM Hepes, 50 mM Tris, 10 mM dextrose, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM KCl, 10 mM $CaCl_2$, 2 mM EDTA,

Table II. Adipocyte Insulin Action and Fold-Stimulation Fibroblast Insulin Receptor Autophosphorylation

Subject	ED_{50} insulin pM	Basal	Max (al/mm²/s)	Percent B/T	Fold
Obese PCOS					
1	415	0.45	1.32	8.9	2.9
2	482	0.58	1.11	3.4	2.4
3	359	1.66	3.53	6.6	1.4
4	98	0.56	0.88	6.8	1.3
5	139	1.53	3.22	6.7	1.1
6	200	0.57	1.07	9.0	1.6
7	87	1.67	3.50	3.0	2.4
8	59	1.39	2.42	2.6	1.6
9	ND	ND	ND	ND	1.0*
10	ND	ND	ND	ND	1.0*
Mean±SEM	230±58	1.05±0.20	2.13±0.41	5.9±0.9	
Obese control n = 4					
mean±SEM	101±29	1.09±0.12	2.35±0.10	7.1±2.0	
Nonobese PCOS					
11	58	0.82	1.48	2.8	2.0
12	244	2.56	7.51	9.0	2.4
13	52	2.95	6.80	10.1	3.4
14	412	0.62	1.31	5.1	2.2
15	29	0.84	1.55	4.5	1.3
16	147	2.79	4.25	7.3	1.1
Mean±SEM	157±60	1.76±0.45	3.82±1.15	6.5±1.2	1.9±0.7**
Nonobese control n = 4					
mean±SEM	122±50	2.5±0.23	6.12±1.73	8.6±1.4	2.8±0.3***

ED_{50} Insulin, ED_{50} adipocyte insulin-stimulated glucose uptake; *al/mm²/s,* attoliters per millimeter² per second; *Basal,* basal rate and *Max,* maximal rate of adipocyte insulin-stimulated glucose uptake; *Percent B/T,* percent bound over total adipocyte [^{125}I]insulin binding; * skeletal muscle insulin receptor; ** insulin-induced fold-stimulation of fibroblast insulin receptor autophosphorylation in obese and nonobese PCOS; *** insulin-induced fold-stimulation of fibroblast insulin receptor autophosphorylation in obese and nonobese controls.

and 1% BSA (pH 8.0) (26). Each concentration of unlabeled insulin was tested in triplicate. Cells were washed with ice cold phosphate buffered saline (pH 7.4) to remove unbound isotope and solubilized in 0.2 N NaOH. Bound [^{125}I]insulin was determined by gamma counting and protein concentrations were determined by the Lowry procedure (26). Scatchard analysis was performed on the binding data using the Ligand computer program. The interassay coefficient of variation was 9% for the fibroblast insulin binding studies. Fibroblast insulin binding data from the control women were reported as normative data in a previous manuscript (26).

Studies of insulin receptor autophosphorylation and protein tyrosine kinase activity. Skin fibroblasts were grown to confluence, serum-starved for 48 h and harvested in 10 mM Hepes, 1 mM EDTA, pH 7.4. After the addition of sucrose to 10% (wt/vol), the cells were lysed by nitrogen cavitation (20 min, 400 psi, 4°C) (cell disruption bomb, Par Instrument Co, Moline, IL) and the nuclei were separated by centrifugation at 1,000 *g* for 10 min at 4°C (27). Plasma membranes were pelleted at 200,000 *g* for 60 min at 4°C and stored at −70°C. Before insulin receptor isolation, fibroblast membranes were washed first with 50 mM Hepes, 1 mM EDTA, pH 7.0 (buffer A) and then with buffer A containing 1.5 M NaCl (27). Membranes then were solubilized in buffer A containing 2% Triton. For studies of insulin receptor protein kinase activity, insulin receptors were extracted directly from confluent serum-starved fibroblasts with 50 mM Hepes, 1 mM EDTA, 2 mM PMSF, 0.1 mg/ml aprotinin, 1% Triton, pH 7.4, at 4°C for 1 h (28). Extracts were then spun at 150,000 *g* for 1 h at 4°C (28). Insulin receptors were partially-purified from solubilized fibroblast membranes and from fibroblast monolayer extracts by affinity chromatography on WGA-Sepharose (column volume 300 μl). For autophosphorylation studies, receptors were eluted with 0.3 M *N*-acetylglucosamine in buffer A containing 0.12% Triton. For substrate phosphorylation studies, fibroblast extracts were eluted with 0.3 M *N*-acetylglucosamine in 50 mM Hepes, 1 mM EDTA, 2 mM PMSF, 0.1 mg/ml aprotinin, 0.12% Triton, pH 7.4. WGA–Sepharose columns were washed four times with 250 μl of elution buffer. The eluates were pooled and concentrated to 100–200 μl with Centricon-30 concentrators. Skeletal muscle homogenates were prepared and insulin receptors were then partially purified by affinity chromatography on 300 μl of WGA–Sepharose as reported (29). WGA–Sepharose columns were washed and pooled eluates were concentrated as described above.

Insulin binding was determined in the lectin eluates in the presence of binding buffer (200 mM Na_2HPO_4, 1 mM EGTA, 1 mM EDTA pH 7.8), 1% bovine serum albumin, [^{125}I] insulin and unlabeled insulin (increasing concentrations or 17.5 μM). The final reaction volume (300 μl) was incubated overnight at 4°C and was precipitated by incubation at 4°C for 15 min with 0.4% bovine gamma globulin and 20% polyethylglycol. Bound [^{125}I] insulin was pelleted in a microcentrifuge at 4°C for 15 min and counts were determined by gamma scintillation. Eluate protein concentration was determined using the Biorad Protein Assay (Hercules, CA) and insulin receptor number and affinity were determined using the Ligand computer program.

Autophosphorylation was examined by incubating WGA–Sepharose eluates at room temperature in the presence or absence of 1 μM insulin (28, 30). Similar amounts of protein (~ 10 μg for fibroblast studies and ~ 20 μg for skeletal muscle studies) were used and PCOS subjects were always assayed in parallel with samples from control women. The studies in partially-purified fibroblast insulin receptors were performed at the optimal pH for the insulin receptor tyrosine kinase, pH 7.0, and 1 μM insulin was used to ensure maximal insulin receptor activation at this pH (27, 30). Studies in skeletal muscle were performed at pH 7.4. Reactions were initiated by the addition of 10 μM ATP, 50 μCi [^{32}P]-γ-ATP (2 μM; 7,000 Ci/mmol sp act) and 5 mM manganese acetate (final concentrations). After 30 min, the reactions were quenched with concentrated Laemmli sample buffer containing 60 mM DTT and analyzed by SDS-PAGE on 8% polyacrylamide gels. The receptor β-subunits were localized on dried gels by autoradiography, excised and counted. Equal areas of each gel judged to be free of proteins were excised and counted as background. Studies were repeated if fold-stimulation was < twofold. Immunochemical identification of receptors was performed by incubation overnight at 4°C with the anti-insulin receptor β-subunit antibody, 18–44 (31), the anti-IGF-I receptor antibody, α-IR3, or the antiphosphotyrosine antibody, PY20, and precipitation with Protein A–Sepharose for 2 h. When immunoprecipitation was performed after autophosphorylation, reactions were first stopped with 50 mM Hepes, 100 mM NaF, 10 mM Na-pyrophosphate, 5 mM EDTA, 2 mM Na-vanadate, pH 7.4, immunoprecipitated overnight and were then analyzed by SDS-PAGE.

Insulin receptor tyrosine kinase activity towards artificial substrate was determined by preincubating 5-μg aliquots of insulin receptors partially-purified from extracts of confluent skin fibroblasts for 30 min at 22°C in 60 μl total volume at pH 7.4 with 0–100 nM insulin to examine insulin receptor kinase activity under physiologic conditions (28). The artificial substrate, poly GLU4:TYRI, (400 μg/40 μl) was added to the reaction volume and phosphorylation was initiated by adding 40 μl of a mixture calculated to give final concentrations of 5 mM manganese acetate, 20 mM $MgCl_2$, 28.6 μM ATP, 2 μCi [^{32}P]-γ-ATP (7,000 Ci/mmol sp act) and 50 mM Hepes, pH 7.4. After incubation for 20 min phosphorylation was terminated by spotting on Whatman # 3 filter paper (28). The papers were washed at room temperature in 10% trichloroacetic acid containing 10 mM Na-pyrophosphate with constant stirring over 24 h and the remaining radioactivity was determined by Cerenkov scintillation counting. Data were expressed as fmol of [^{32}P] phosphate incorporated per mg of substrate per minute per fmol insulin binding.

Phosphoamino acid analysis. Phosphorylated β-subunits of the insulin receptor were identified by their mobility on SDS-gels. Immunoprecipitation after autophosphorylation with the anti-insulin receptor antibody 18–44 confirmed the identity of the insulin receptor β-subunit but was not performed routinely to ensure that sufficient amounts of phosphorylated β-subunit remained in both the absence and presence of insulin for phosphoamino acid analysis. β-subunits were cut from dried gels according to properly aligned autoradiograms, rehydrated in water and digested overnight in 50 mM *N*-ethylmorpholine acetate, pH 8.6 containing 0.1 mg/ml trypsin. Lyophilized tryptic peptides were hydrolyzed with 6 N HCL containing 1 mg/ml phenol for 30 min at 150°C (32), and [^{32}P]phosphoamino acid were separated by a thin layer high voltage electrophoresis at pH 3.5 using a flatbed gel apparatus, Hoefer Scientific (San Francisco, CA) (32). Phosphoamino acids were quantitated by scraping appropriate regions of thin layer plates and subjecting each sample to Cerenkov scintillation counting for 30 min.

Mixing experiments. The NIH 3T3 fibroblasts overexpressing the human insulin receptor (hIR) cDNA were grown to confluence and serum-starved as described above. Plasma membranes were prepared and hIRs were partially-purified by affinity chromatography on WGA-Sepharose prior to further purification by immunoprecipitation with the anti-insulin receptor antibody, 18–44 as described above. Immunopurified hIRs were then mixed with WGA–Sepharose eluates from PCOS fibroblast membranes containing insulin receptors with increased phosphoserine-decreased phosphotyrosine (PCOS-Ser) or from control fibroblasts in a ratio of ~ 10 fmol hIR:1 fmol WGA-Sepharose insulin binding activity. Autophosphorylation in the presence and absence of 1 μM insulin was then performed as described above. Phosphorylated β-subunits were excised from SDS gels and phosphoamino acid analysis was performed as outlined above.

In vivo fibroblast labeling. Control and PCOS-Ser fibroblasts (7–11th passage) were maintained in supplemented DME media as described above. Confluent cells were serum starved with DME containing 0.1% BSA for 48 h and pre-incubated in serum-free and phosphate-free DME media with 10 mM Hepes, pH 7.5 for 1 h at 37°C, labeled with 0.4 mCi/ml of [^{32}P]orthophosphate for 4 h, and incubated in the presence and absence of 100 nM insulin for 10 min (33). Reactions were terminated by removing the media and immediately freezing cells in liquid nitrogen. Cells were solubilized and cell lysates prepared as reported (33). Equal protein concentrations of cell lysates (800 μg) were incubated overnight at 4°C with 4 μg of the polyclonal anti-insulin receptor antibody and immunoprecipitated complexes were resolved as above by SDS-PAGE.

Denaturing gradient gel electrophoresis (DGGE). Genomic DNA was extracted from blood or confluent skin fibroblasts and amplified by PCR as reported (26, 34). Amplified fragments of DNA were analyzed by parallel DGGE in a Hoefer SE600 vertical apparatus at 60°C as reported (26, 34). Perpendicular DGGE was performed when a potential mutation was detected (26, 34). Amplified DNA was directly sequenced as reported (26) when the perpendicular DGGE suggest the presence of a bp change.

Data analysis. Fibroblast insulin binding and phosphorylation data from obese and nonobese individuals in the PCOS and the control groups were combined since changes in these parameters related to obesity reflect the in vivo hormonal environment and resolve in tissue culture (11–13, 26, 35). Two groups were analyzed by unpaired *t* tests. When three groups were present (i.e., PCOS-Ser, PCOS-n1, control), data analysis was performed by one-way ANOVA with Tukey's post-hoc test to determine which group(s) differed significantly. Simple linear regression analyses were performed between parameters of insulin receptor phosphorylation and adipocyte insulin action. Log transformation of the data was performed when necessary to achieve homogeneity of variance. Statistical analyses were performed using SAS software (Cary, NC) and all data are reported as the untransformed mean±SEM.

Results

Autophosphorylation studies. There were no apparent differences in receptor number or affinity by Scatchard analysis (data not shown). Partially-purified PCOS insulin receptors were recognized by anti–β-subunit monoclonal antibody 18–44 (Fig. 1). PCOS β-subunits of insulin and IGF-I receptors, labeled by autophosphorylation, migrated in SDS-gels with an apparent molecular weight similar to the control β-subunits (Fig. 1). No functional insulin receptors from either PCOS or control women were found in the nonadsorbed flow-through of the lectin column. Insulin-stimulated autophosphorylation of partially purified fibroblast insulin receptors was significantly decreased in PCOS (fold stimulation±SEM [1.0 = no stimulation] 1.9±.7 PCOS vs 2.8±.3 control, $P < 0.05$, Table II).

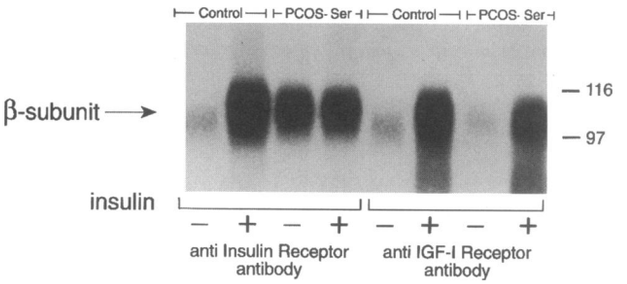

Figure 1. Autoradiogram of insulin receptor β-subunits immunoprecipitated with anti-insulin receptor antibody and of IGF-I receptor β-subunits immunoprecipitated with anti–IGF-I receptor antibody after autophosphorylation±1 μM insulin. Aliquots of partially purified skin fibroblast insulin and IGF-I receptors from a PCOS-Ser and a control woman containing equal amounts of protein (10 μg) were incubated in the presence or absence of 1 μM insulin as indicated and the autophosphorylation reactions were terminated after 30 min as described in Methods. Autophosphorylated receptors were immunoprecipitated for 2 h with protein A–Sepharose after incubation overnight at 4°C with specific antibodies to the insulin and IGF-I receptors, reduced with DTT and resolved by SDS-PAGE. The insulin receptor β-subunit (*left panel*) from the PCOS-Ser has marked phosphate incorporation in the absence of insulin and minimal stimulation of phosphate incorporation by 1 μM insulin as compared to the control insulin receptor β-subunit. In contrast, basal and stimulated (by spillover occupancy by insulin) IGF-I receptor β-subunit phosphate incorporation is similar in PCOS-Ser and in control (*right panel*).

There appeared to be two groups of PCOS insulin receptors. Insulin receptors from seven of 14 PCOS women (50%) and one normal woman had increased basal autophosphorylation (> 60% of insulin-stimulated autophosphorylation) (Fig. 2). The other 7 PCOS women had basal autophosphorylation (< 50% of insulin-stimulated phosphorylation) and insulin-stimulated receptor autophosphorylation similar to receptors from control women (Fig. 2). This was investigated a priori by re-establishing skin fibroblast cultures from these cell lines and performing additional studies with partially purified insulin receptors. In the PCOS insulin receptors with increased basal autophosphorylation (PCOS-Ser, $n = 7$), basal [^{32}P]phosphate incorporation was increased significantly (3.7-fold, one-way ANOVA $P < 0.001$, $P < 0.05$ vs. other groups by Tukey's test) and there was minimal insulin-stimulated phosphorylation (Fig. 3). In PCOS insulin receptors with normal basal autophosphorylation (PCOS-n1, $n = 4$), basal and insulin-stimulated [^{32}P]phosphate incorporation was similar to that seen in control insulin receptors (Fig. 3). There were no significant correlations between parameters of receptor phosphorylation and insulin action in PCOS and control women. There were no significant differences in clinical characteristics or parameters of insulin

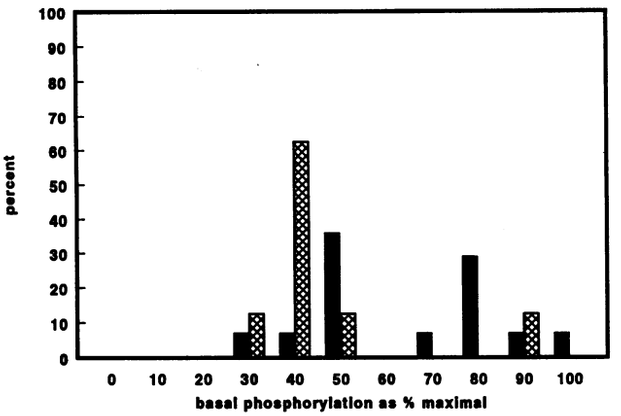

Figure 2. The distribution of basal skin fibroblast insulin receptor autophosphorylation in PCOS (*solid bars*) and control women (*cross-hatched bars*). Basal receptor autophosphorylation is represented as the percent of maximal insulin-stimulated autophosphorylation. There appear to be two groups of insulin receptors from PCOS women. 50% of PCOS insulin receptors have high basal phosphate incorporation > 60% and ~ 50% of PCOS insulin receptors have basal phosphate incorporation < 50% of maximal autophosphorylation, similar to that seen in controls.

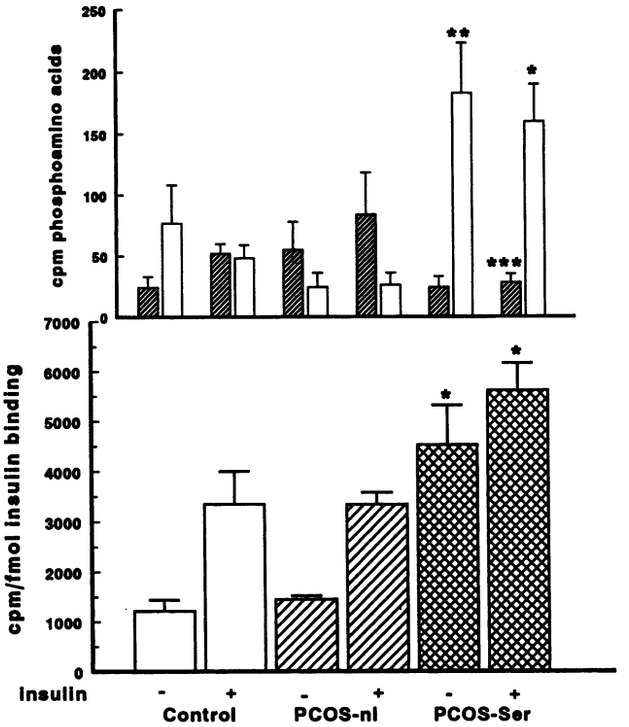

Figure 3. Phosphate incorporation in insulin receptor β-subunits partially-purified from skin fibroblast membranes of control (*open bars*), PCOS-n1 (*hatched bars*) and PCOS-Ser (*cross-hatched bars*) women with phosphoamino acid analysis shown in the inset. The β-subunits were phosphorylated in the presence and absence of 1 μM insulin, resolved by SDS-PAGE, excised from the gel, counted and phosphoamino acid analysis performed as described in Methods. β-subunit [^{32}P]-phosphate incorporation±1 μM is significantly increased in PCOS-Ser vs other groups; one-way ANOVA basal $P < 0.001$, + 1 μM insulin $P < 0.05$; * $P < 0.05$ vs control and PCOS-n1 by Tukey's post hoc test. The values are the mean±SEM from 7 PCOS-Ser, 4 PCOS-n1, and 6 control subjects. The cpms tyrosine (*cross-hatched bars*) and serine (*open bars*)±1 μM insulin after β-subunit phosphoamino acid analysis are shown in the inset; one-way ANOVA basal tyrosine P = NS, serine $P < 0.05$, + 1 μM insulin tyrosine $P < 0.05$, serine $P < 0.001$; * $P < 0.05$ vs control and PCOS-n1, ** $P < 0.05$ vs PCOS-n1 by Tukey's post-hoc test; *** $P < 0.05$ for three groups by one-way ANOVA. The values are the mean±SEM from 7 PCOS-Ser, 3 PCOS-n1, and 5 control subjects.

action in the PCOS-Ser compared to the PCOS-n1 women (data not shown). One normal control woman also had increased basal autophosphorylation. She was nonobese and had normal adipocyte insulin action. Unfortunately, her fibroblast lines were not available for further studies.

Phosphoamino acid analysis of the insulin receptor β-subunits revealed that [^{32}P]phosphate incorporation into serine residues was elevated in PCOS-Ser compared to controls (one-way ANOVA $P < 0.05$, $P < 0.05$ vs PCOS-n1 by Tukey's test) and insulin-stimulated tyrosine phosphorylation was diminished (one-way ANOVA $P < 0.05$, P = NS vs other groups by Tukey's test) (Figs. 3 and 4). Insulin-independent phosphoserine content and insulin-dependent phosphotyrosine content were similar to control levels in PCOS-n1 β-subunits (Fig. 3 and 4). No phosphothreonine was detected. Immunoprecipitation after autophosphorylation confirmed that the abnormal pattern of phosphorylation was present in the insulin receptor β-subunit (Fig. 1). However, insulin induced (by spillover occupancy) similar patterns of [^{32}P]phosphate incorporation in PCOS-Ser and control IGF-I receptors (Fig. 1) and antiphosphotyrosine antibody immunoprecipitated similar amounts of β-subunit under these conditions to that immunoprecipitated by αIR-3 antibody (data not shown) suggesting that the PCOS-Ser IGF-I receptor tyrosine phosphorylated normally.

Insulin receptors partially-purified from PCOS skeletal muscle and immunoprecipitated after autophosphorylation had the same increased insulin-independent β-subunit [^{32}P]phosphate incorporation and minimal further insulin-stimulated phosphorylation as that seen in PCOS skin fibroblast insulin receptors (Fig. 5). Phosphoamino acid analysis revealed that this increased phosphate incorporation was into serine rather than tyrosine residues (data not shown). Insulin receptor β-subunits phosphorylated in vivo in PCOS-Ser fibroblasts showed the same pattern of phosphorylation as that found in insulin receptors partially-purified from these cells and phosphorylated in vitro (data not shown). Patterns of insulin receptor autophosphorylation also did not differ in control fibroblast receptors phosphorylated in vitro and in vivo (data not shown). The presence of this abnormal pattern of receptor phosphorylation in insulin receptors isolated from [^{32}P]phosphate labeled intact cells and from skeletal muscle, the major insulin target tissue, suggests that this finding is not an artifact of the phosphorylation conditions or tissue used.

Artificial substrate phosphorylation studies. Tyrosine kinase activity of the partially purified skin fibroblast insulin receptors was determined by the ability of the receptors to phosphorylate the artificial substrate poly GLU4:TYR1. Basal rates of substrate phosphorylation were similar in PCOS-Ser, PCOS-n1 and control women (Fig. 6). Maximal rates of substrate phosphorylation as well as the insulin-induced increment over basal were significantly decreased in PCOS-Ser compared to control and PCOS-n1 insulin receptors (both one-way ANOVA $P < 0.005$, $P < 0.05$ vs other groups by Tukey's test, Fig. 6).

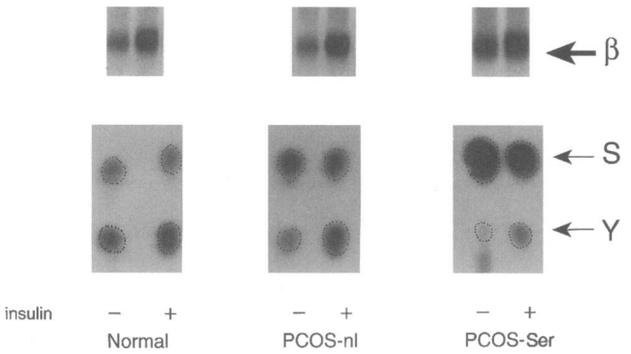

Figure 4. Representative autoradiograms of autophosphorylated skin fibroblast insulin receptor β-subunits (*top*) and phosphoamino acid analysis (*bottom*) ±1 μM insulin from normal (control), PCOS-nl and PCOS-Ser women; S-serine, Y-tyrosine. Autophosphorylation and phosphoamino acid analysis were performed as described in Methods.

Immunopurification and mixing studies. When partially purified insulin receptors were immunoprecipitated with anti-insulin receptor antibody, 18–44, before autophosphorylation, the amount of basal and insulin-stimulated [^{32}P]phosphate incorporation into PCOS-Ser insulin receptors was similar to control (Table III). Both control and PCOS-Ser receptors had greater fold-stimulation of autophosphorylation after immunoprecipitation. This finding suggests that these isolated receptors did not contain increased amounts of insulin-independent phosphoserine. Mixing of immunopurified hIR with PCOS-Ser skin fibroblast WGA–Sepharose eluate resulted in increased insulin-independent β-subunit phosphorylation on serine residues and decreased insulin-stimulated tyrosine phosphorylation as compared to hIR mixed with control skin fibroblast WGA-Sepharose eluate and to unmixed hIR (Fig. 7).

Insulin receptor gene sequencing. We have previously reported direct DNA sequencing of the insulin receptor gene in two of the PCOS-Ser women (subjects 6 and 8, Tables I and II) that showed no mutations and both alleles appeared to be

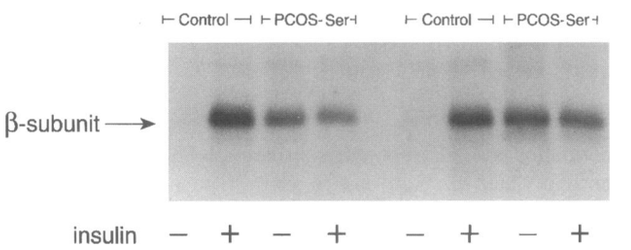

Figure 5. Autoradiogram of skeletal muscle insulin receptor β-subunits immunoprecipitated with anti-insulin receptor antibody, 18–44, after autophosphorylation±1 μM insulin. Aliquots of partially-purified skeletal muscle insulin receptors from 2 PCOS-Ser and 2 control women containing equal amounts of protein (20μg) were incubated in the presence or absence of 1 μM insulin and the autophosphorylation reactions terminated after 30 min as described in Methods. The autophosphorylated receptors were immunoprecipitated and resolved by SDS-PAGE as described in Fig. 1. There is increased basal autophosphorylation in both PCOS β-subunits and no further stimulation of phosphorylation by insulin (cpm basal/insulin 763/503, 967/901, respectively). In the control insulin receptors, there is minimal insulin-independent autophosphorylation and 10–14-fold insulin-stimulated phosphorylation (cpm basal/insulin 98/1414, 96/1036, respectively).

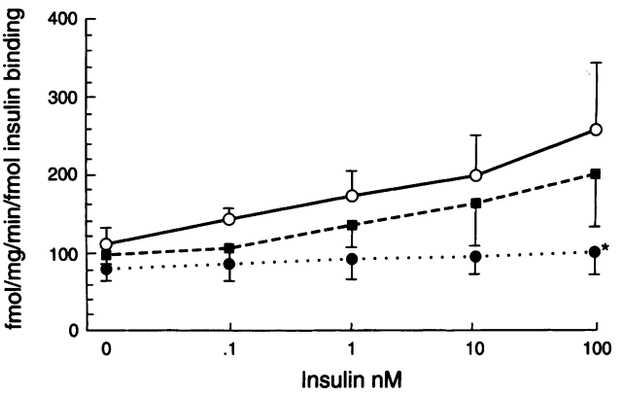

Figure 6. Phosphorylation of poly GLU4:TYR1 by aliquots (5 μg protein) of partially-purified skin fibroblast insulin receptors at pH 7.4, in the presence of 0-100 nM insulin, *1-way ANOVA $P < 0.005$; PCOS-Ser < control and PCOS-nl, $P < 0.05$ Tukey's test. Skin fibroblast insulin receptors were directly extracted from confluent cell cultures, partially-purified, incubated in the presence of 0–100 nM and assays of the phosphorylation of poly GLU4:TYR1 performed as outlined in Methods. The values are the mean±SEM from 5 PCOS-Ser (●), 4 PCOS-nl (■), and 4 control (○) subjects.

expressed normally (26). The remaining PCOS-Ser women were screened for mutations in the insulin receptor β-subunit exons containing tyrosine phosphorylation sites (exons 16–22) by DGGE and direct sequencing, when necessary. No mutations were detected.

Discussion

Cellular insulin action is initiated by insulin binding to the α-subunit of the receptor (for review see reference 22). This results in autophosphorylation of the β-subunit on tyrosine residues thereby increasing the receptor's protein tyrosine kinase activity. The autophosphorylated β-subunit then tyrosine phosphorylates endogenous substrates, such as insulin receptor substrate-1, resulting in a cascade of intracellular signaling events. The mechanisms for terminating the insulin signal have not been identified. However, serine phosphorylation of the insulin receptor in vivo and in cell-free systems has been shown to decrease the receptor's protein tyrosine kinase activity and may, thus, represent one mechanism for terminating insulin signaling (19–22, 36, 37). Defects in insulin receptor autophosphorylation or substrate phosphorylation could result in the abnormalities of cellular insulin action characteristic of PCOS (6, 7).

Table III. Immunoprecipitation of Fibroblast Insulin Receptors After and Before Autophosphorylation

Subject	− Insulin cpm	+ Insulin cpm	Fold
After			
Control	237	2180	9.2
PCOS-Ser	1128	1156	1.0
Before			
Control	603	4290	7.1
PCOS-Ser	265	1828	7.0

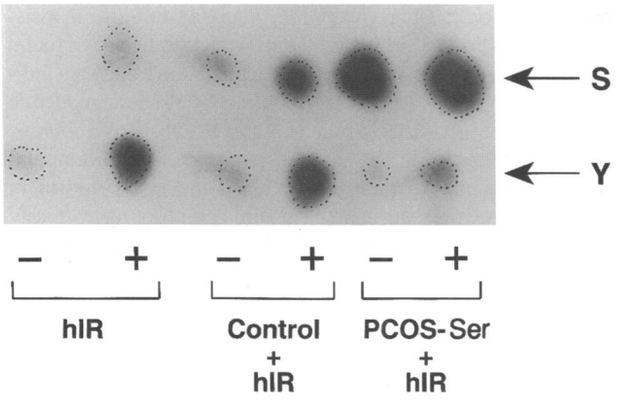

Figure 7. Phosphoamino acid analysis of immunopurified hIR β-subunits, basally and mixed with WGA–Sepharose eluates from control or PCOS-Ser fibroblasts. hIRs were immunopurified from WGA-Sepharose eluates with anti-insulin receptor antibody, 18–44, and mixed in a ratio of 10 fmol hIR: 1 fmol PCOS-Ser or control lectin eluate insulin binding activity. The β-subunits were autophosphorylated resolved by SDS-PAGE, localized by autoradiography, excised and hydrolyzed as described in Methods. Autophosphorylation±1 μM insulin of immunopurified hIR showed 63/234 cpm/fmol basal insulin and hIR mixed with control lectin eluate showed 59/210 cpm/fmol basal/insulin, whereas hIR mixed with PCOS-Ser showed 249/474 cpm/fmol basal/insulin. Phosphoamino acid analysis revealed a striking increase in phosphoserine content and a marked decrease in insulin-stimulated phosphotyrosine content after mixing with PCOS-Ser lectin eluates as compared to mixing with control lectin eluates or in the absence of mixing.

As expected from previous studies in isolated adipocytes (6, 7), there were no significant decreases in the number or affinity of cell surface insulin binding sites in skin fibroblasts from PCOS women. There was a modest, ~ 30%, but significant decrease in insulin-induced fold-stimulation of autophosphorylation in PCOS as compared with control insulin receptors. When the individuals were examined, however, there appeared to be two groups of PCOS insulin receptors: one with increased basal phosphorylation and minimal further insulin-stimulated autophosphorylation, and one with basal and insulin-induced phosphorylation similar to control insulin receptors. To explore this hypothesis a priori skin fibroblast cultures were re-established and insulin receptor phosphorylation was examined in each group of PCOS insulin receptors. We found significantly different patterns of [^{32}P]phosphate incorporation in the PCOS-Ser receptors which had increased [^{32}P]phosphate incorporation in the absence of insulin and minimal net stimulation of incorporation by insulin. In contrast, β-subunit [^{32}P]phosphate incorporation in the absence and presence of insulin was similar in the PCOS-n1 and the control receptors.

Phosphoamino acid analysis of the β-subunits revealed that [^{32}P]phosphate incorporation was significantly increased in serine residues in PCOS-Ser β-subunits in the absence of insulin. Phosphoserine content was not further increased by insulin and there was significantly decreased insulin-stimulated [^{32}]phosphate incorporation into tyrosine residues in the PCOS-Ser β-subunits. The PCOS-n1 and control β-subunits had similar amounts of insulin-stimulated tyrosine phosphorylation. Phosphoserine was also present basally and demonstrated no stimulation by insulin in PCOS-n1 and control women. Immunoprecipitation with the specific anti-insulin receptor monoclonal antibody after autophosphorylation confirmed that the abnormal pattern of basal phosphorylation was present in the insulin receptor β-subunit. The autophosphorylation of the IGF-I receptor induced by the high concentrations of insulin used (Fig. 1) indicated that some of the [^{32}P]phosphate incorporation was present in the β-subunits of the IGF-I receptor in the receptor preparations that were not immunopurified after autophosphorylation. Since human skin fibroblasts contain both insulin and IGF-I receptors (38, 39), it is probable that hybrid insulin/IGF-I receptors were present and that their phosphorylated β-subunits were immunoprecipitated by both the anti-insulin receptor and the anti–IGF-I receptor antibodies (40, 41). The presence of these receptors does not alter the conclusion that PCOS-Ser insulin receptor β-subunits contained increased phosphoserine because immunoprecipitated IGF-I receptors had minimal basal [^{32}P]-incorporation (Fig. 1) and immunoprecipitation with antiphosphotyrosine antibody yielded similar results. This suggests that the αIR-3 immunoprecipitated β-subunits were tyrosine phosphorylated. It remains possible that some basal phosphoserine was present in hybrid receptor β-subunits (40, 41).

The ability of partially purified insulin receptors to stimulate phosphorylation of the artificial substrate poly GLU4:TYR1 was examined over a range of physiologic insulin concentrations. The maximal rates of substrate phosphorylation as well as the increment in the rate of phosphorylation over basal were significantly decreased in insulin receptors from PCOS-Ser compared to insulin receptors from PCOS-n1 and control women. Insulin receptors from PCOS-n1 and control women did not differ significantly in their ability to phosphorylate artificial substrate. Thus, increased insulin receptor serine phosphorylation was associated with decreased receptor tyrosine kinase activity with respect to both tyrosine autophosphorylation and substrate phosphorylation.

The increased insulin-independent insulin receptor phosphorylation did not differ for PCOS-Ser receptors phosphorylated *in vivo* (42). The abnormal pattern of insulin receptor phosphorylation was also present in insulin receptors partially purified from PCOS skeletal muscle. Taken together, these findings indicate that this phosphorylation abnormality can occur in intact cells and in the major insulin target tissue, muscle, supporting the hypothesis that excessive insulin receptor serine phosphorylation may cause insulin resistance in affected PCOS women. Nevertheless, only artificial substrate was examined and the ability of the insulin receptor to phosphorylate endogenous substrates may differ (21). Studies of activation of endogenous insulin receptor substrates are currently in progress in our laboratory to determine whether the changes in insulin receptor phosphorylation reported here are physiologically relevant.

Defects in receptor autophosphorylation have been reported with a variety of mutations in the insulin receptor gene (for a review see reference 43). Increased insulin-independent [^{32}P]-phosphate incorporation has been reported occasionally with insulin receptor mutations (44–47). These latter receptors have been constituitively active, however, and have been tyrosine phosphorylated (44–47). In two PCOS-Ser women in whom we have previously directly sequenced the entire coding portion of the insulin receptor gene, no mutations or evidence for decreased expression of one allele was found (26). The remaining PCOS-Ser women had no mutations detected by DGGE screening of exons 16–22 of the insulin receptor gene, representing the exons containing the β-subunit tyrosine phosphorylation

sites (43). It remains possible that mutations, such as those that alter the expression of one allele of the insulin receptor, escaped detection, although such mutations usually produce marked insulin binding defects and only occasionally affect receptor autophosphorylation (43, 48).

Immunoprecipitation of the PCOS-Ser insulin receptors prior to autophosphorylation resulted in normal fold-stimulation of autophosphorylation and [^{32}P]phosphate incorporation. Mixing PCOS-Ser WGA-eluates with a 10-fold excess of control hIR resulted in increased insulin-independent serine phosphorylation and decreased insulin-stimulated tyrosine phosphorylation of these insulin receptors. These results strongly suggest that the WGA-eluates contained a factor that was responsible for the excessive serine phosphorylation of the PCOS-Ser insulin receptors. This factor could be either a serine/threonine kinase or an inhibitor of a serine/threonine phosphatase (49). Since the PCOS-Ser IGF-I receptors had [^{32}P]phosphate incorporation similar to control, this suggests that the insulin receptor but not the IGF-I receptor was a substrate for this factor. Thus, serine residues unique to the insulin receptor β-subunit may have been the sites of phosphorylation (50, 51). Mapping of the β-subunit phosphorylation sites will be necessary to investigate this hypothesis.

Candidate factors include an isoform of protein kinase C, a casein kinase I-like enzyme and cyclic AMP kinase (21, 52, 53). There is extensive evidence indicating that protein kinase C can serine phosphorylate the insulin receptor (19–21). Indeed, overexpression of protein kinase Cα and human insulin receptors in Chinese hamster ovary cells results in decreased in vivo insulin signaling coincident with protein kinase C–mediated insulin receptor serine phosphorylation (21). Arguing against this, we detected no phosphothreonine and protein kinase-C has been shown to phosphorylate threonine 1336 of the insulin receptor ([54] numbered according to Ullrich et al. reference 50). Further, the IGF-I receptor also appears to be a substrate for this kinase (55). Alternatively, a novel factor may be responsible for the excessive serine phosphorylation. Insulin-stimulated serine phosphorylation of the insulin receptor also occurs and this appears to be secondary to intrinsic insulin receptor serine kinase activity (56, 57). However, in the present study insulin receptor serine phosphorylation was insulin-independent and the serine phosphorylation factor was extrinsic to the receptor indicating that the receptor was not constituitively active.

Recent studies have identified an inhibitor of insulin receptor tyrosine kinase activity in WGA-eluates of skin fibroblasts from some insulin resistant subjects (58–60). This inhibitor, the membrane glycoprotein PC-1, differs from our putative serine phosphorylation factor in that it does not cause the increased basal [^{32}P]phosphate incorporation characteristic of the serine phosphorylated PCOS-Ser insulin receptors (58, 60). Further, PC-1 appears to be a threonine-specific protein kinase (61). Many, but not all, of the insulin resistant patients examined had increased PC-1 activity, similar to our finding that only 50% of PCOS women had increased insulin receptor serine phosphorylation. Other causes for insulin resistance must be sought in these individuals. Both studies, however, suggest an important role for factors extrinsic to the insulin receptor that regulate its kinase activity in the pathogenesis of human insulin resistance.

This is the first report, to the best of our knowledge, of excessive insulin receptor serine phosphorylation associated with a human disorder of insulin action. However, it has been suggested that hyperglycemia induces insulin receptor serine phosphorylation causing a reversible component of insulin resistance in NIDDM (62, 63). Limited previous studies have found minimal phosphoserine in insulin receptor β-subunits from individuals with NIDDM (64, 65). In contrast, we found a moderate amount of phosphoserine in control and PCOS-n1 β-subunits. Technical differences in the autophosphorylation conditions and reaction times may explain this observation since studies in cell lines overexpressing human insulin receptors have clearly shown that these receptors are serine phosphorylated under various conditions (21, 53, 57). The presence of phosphoserine, in addition to phosphotyrosine, under normal circumstances raises the possibility that the insulin receptor may be under tonic inhibition by serine phosphorylation and that the factor responsible for this may be upregulated in PCOS-Ser cells. Serine dephosphorylation studies will be required to address this issue. The presence of increased phosphoserine in hIR β-subunits after mixing with control lectin eluates suggests that the serine phosphorylation factor is present in normal human fibroblasts but not in murine fibroblasts (Fig. 7).

The excessive serine phosphorylation appears to be a genetic defect since it persists in cultured cells (43). Although skin fibroblasts are not classic insulin target tissues, abnormalities in insulin binding and receptor autophosphorylation identified in fibroblasts have reflected mutations in the insulin receptor gene (43–45, 47). Moreover, we have also found increased phosphoserine and decreased phosphotyrosine in insulin receptors isolated from PCOS muscle, the major insulin target tissue. We hypothesize that the factor producing excessive insulin receptor serine phosphorylation is a likely locus for a mutation producing the insulin resistant state in affected PCOS women. Whether the decrease in adipocyte GLUT4 content (8) is an associated genetic defect or an acquired abnormality secondary to decreased insulin receptor-mediated signaling will require further study. There were no apparent phenotypic differences in the PCOS-Ser compared with the PCOS-n1 women; both groups were insulin resistant, had Caucasian and Hispanic as well as lean and obese individuals. We hypothesize that the PCOS-n1 women will have downstream defects in insulin receptor signaling since they have a similar biochemical phenotype to that of the PCOS-Ser women, i.e., a striking loss of insulin sensitivity (6, 7, 22). PCOS is the first common insulin resistant state conferring an increased risk for NIDDM in which a specific and, potentially, intrinsic biochemical defect in insulin receptor signal transduction appears to be present (66).

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