Defects in insulin receptor signaling in vivo in the polycystic ovary syndrome (PCOS)

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Dunaif, Andrea, Xinqi Wu, Anna Lee, and Evanthia Diamanti-Kandarakis. Defects in insulin receptor signaling in vivo in the polycystic ovary syndrome (PCOS). Am J Physiol Endocrinol Metab 281: E392–E399, 2001.—Women with polycystic ovary syndrome (PCOS) are insulin resistant secondary to a postbinding defect in insulin signaling. Sequential euglycemic glucose clamp studies at 40 and 400 mU·m−2·min−1 insulin doses with serial skeletal muscle biopsies were performed in PCOS and age-, weight-, and ethnicity-matched control women. Steady-state insulin levels were significantly decreased in PCOS women (P < 0.05). Insulin receptor substrate (IRS)-1-associated phosphatidylinositol 3-kinase (PI 3K) activity was significantly decreased in PCOS (n = 12) compared with control skeletal muscle (n = 8; P < 0.05). There was no significant difference in the abundance of IR, IRS-1, or the p85 regulatory subunit of PI 3K in PCOS (n = 14) compared with control (n = 12) muscle (P > 0.05). The abundance of IRS-2 was significantly increased (P < 0.05) in PCOS skeletal muscle, suggesting a compensatory change. We conclude that there is a physiologically relevant defect in insulin receptor signaling in PCOS that is independent of obesity and type 2 diabetes mellitus.

insulin resistance; signal transduction; phosphatidylinositol 3-kinase; insulin receptor substrate-1; insulin receptor substrate-2

POLYCYSTIC OVARY SYNDROME (PCOS) is among the most common endocrine disorders of premenopausal women, affecting ~5% of this population (9, 26). Its etiology is unknown, and it is diagnosed by the presence of hyperandrogenism and chronic anovulation with the exclusion of specific conditions of the adrenal, ovary, or pituitary (9–13). PCOS is also associated with profound peripheral insulin resistance, conferring an increased risk for the development of type 2 diabetes mellitus (DM) at an early age (9, 11, 28). In PCOS, there is a marked shift to the right in the dose-response curve for insulin-mediated glucose use with normal insulin receptor number and affinity (5, 12). Maximal responses to insulin are more modestly, but significantly, decreased (12, 34). These findings suggest that insulin receptor-mediated signal transduction is defective (5, 12). Our studies of insulin receptors partially purified from cultured PCOS skin fibroblasts have shown a significant decrease in insulin-stimulated receptor autophosphorylation, the first step in insulin signal transduction (13). This appears to be secondary constitutive serine phosphorylation of the receptor and decreased insulin-stimulated tyrosine phosphorylation (13). The serine-phosphorylated receptors have significantly decreased kinase activity toward artificial substrate (13). Immunopurification and mixing studies suggest that a factor extrinsic to the receptor, presumably a serine kinase, causes the abnormal pattern of phosphorylation in PCOS fibroblasts (13).

Insulin stimulation of glucose transport occurs via insulin receptor tyrosine phosphorylation of endogenous substrates, such as insulin receptor (IR) substrates (IRSs)-1 and -2 (32, 36, 38). The tyrosine-phosphorylated sites on IRSs serve as docking sites for proteins containing Src-homology 2 domains, including the 85-kDa regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI 3K) (32, 36, 38). Association with the tyrosine-phosphorylated sites on IRS-1/2 results in activation of PI 3K (16, 32, 36, 38). There is now considerable evidence in cells and in rodent tissues to suggest that this signaling pathway subserves GLUT-4 translocation leading to insulin-mediated glucose uptake (3, 16, 23, 31, 32). Clinical studies indicate that PI 3K activation is decreased in association with decreased insulin-stimulated glucose uptake in obesity (17) and in type 2 DM (1, 6, 25, 27), consistent with a role for this signaling pathway in mediating glucose uptake in human skeletal muscle.

If insulin receptor kinase activity is diminished in PCOS, this should result in decreased tyrosine phosphorylation of IRS-1/2, decreased activation of PI 3K, and decreased insulin-mediated glucose uptake (32, 36, 38). This study was performed to determine whether there was evidence for such a defect in the major insulin target tissue, skeletal muscle (8), under physiological circumstances. To accomplish this, we investi-
gated insulin-stimulated glucose uptake and receptor-mediated signaling events in PCOS compared with age-, ethnicity-, and weight-matched control women under euglycemic conditions.

METHODS

Subjects

The study was approved by the Institutional Review Boards of the Pennsylvania State University College of Medicine and the Brigham and Women’s Hospital, and all subjects gave written, informed consent. Fourteen women with PCOS and 12 age-, weight-, and ethnicity-matched control women were studied. Women were 19–41 yr old and in excellent health, and none were taking any medications known to affect carbohydrate or sex hormone metabolism ≥1 mo before study, except for oral contraceptive agents, which were stopped 3 mo before study. No control women had hirsutism, acne, hypertension, or a first-degree relative with type 1 or type 2 DM. None of the women engaged in regular aerobic exercise. All control women had regular menstrual cycles every 27–35 days and normal plasma androgen levels. The PCOS women had irregular menstrual cycles (≥6 months/year) and an elevated total testosterone (T) and/or non-sex-hormone-binding globulin-bound testosterone (uT) level (9–13). Nonclassical congenital adrenal hyperplasia, androgen-secreting neoplasms, and hyperprolactinemia were excluded by appropriate tests in the PCOS women (9–13). All women underwent a 75-g glucose load oral glucose tolerance test in the morning after an overnight fast and a 3-day 300 g/day carbohydrate preparatory diet. Glucose and insulin levels were obtained every 30 min for 2 h, and World Health Organization (WHO) criteria were used to assess glucose tolerance (30).

Experimental Design

All subjects consumed a 300-g/day carbohydrate weight-maintaining diet for 3 days before study and were studied in the morning after a 10- to 12-h overnight fast. Studies were performed in the follicular phase of the menstrual cycle on cycle days 2–11 in the control women and at any time in the PCOS women, all of whom were chronically anovulatory. A sequential two-dose hyperinsulinemic euglycemic clamp was performed as previously described (11, 12). In brief, a catheter was placed in a retrograde fashion in a hand vein for blood sampling and kept heated at 60°C to allow for the collection of arterialized blood. A second catheter was placed in an antecubital vein for infusion of glucose and insulin. After determination of baseline glucose and insulin levels, a primed continuous infusion of insulin at 40 mU·m−2·min−1 was started. After 90 min, the infusion of insulin was increased to 400 mU·m−2·min−1 and continued for 90 min (where + refers to no. of minutes after increased dose). Arterialized blood for plasma glucose determinations was obtained every 5 min throughout the study, and adjustments were made as necessary in the rate of infusion of a 20% dextrose solution to maintain euglycemia. Plasma insulin levels were determined every 15 min.

Under sterile conditions, with the use of 1% lidocaine for local anesthesia, an ~5-mm incision was made in the vastus lateralis muscle ~6 cm above the patella. Percutaneous muscle biopsies were obtained using a 6-mm Bergstrom needle with suction applied (14). Initial studies were performed in three PCOS and six control women with muscle biopsies at 0 and 90 min of each insulin dose. Insulin-mediated glucose uptake (IMGU) was significantly decreased at the higher insulin dose (P < 0.05) and almost achieved significance at the lower insulin dose (P = 0.07) (data not shown). Despite this, there was no significant difference in IRS-1-associated PI 3K activity (data not shown). These observations, taken together with information on the time course of insulin receptor signaling events in animals (19) and human skeletal muscle (17, 20, 27), led us to examine PI 3K activation at biopsies taken at 5, 10, 15, and 30 min after the initiation of each insulin dose in an additional PCOS and a control woman. Maximal changes in IRS-1-associated PI 3K activity were observed at +15 min, and activity had decreased by +30 min in PCOS women. On this basis, biopsies were obtained basally immediately before initiation of the insulin infusion and at 15 and 30 min of the first insulin dose, before the increase to the second insulin dose (90 min), and again at +15, +30, and +90 min during the second dose in the 12 PCOS and 8 control women. Data from two PCOS and four control women who had muscle biopsies at baseline, 90 min, and +90 min were included in the studies of insulin-signaling protein abundance. Several biopsies were obtained from the same incision. All muscle samples were immediately processed for determinations of IRS-1-associated PI 3K activity, and an aliquot was frozen in liquid N2 for immunoblotting.

Assays

Hormone and glucose determinations. T, uT, and dehydroepiandrostrostone sulfate (DHEAS) levels were measured as reported (10). Plasma glucose levels were determined by the glucose oxidase technique with a Beckman Glucose Analyzer 2 (Fullerton, CA), as reported (11, 12). Insulin levels were measured by radioimmunoassay with a Linco Kit (St. Charles, MO).

PI 3K assays (2). All materials were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical (St. Louis, MO), unless otherwise stated. Muscle was placed in 4°C 50 mM HEPES (pH 7.5) ice-cold buffer containing 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% NP-40, 2 mM Na2VO4, 10 mM Na2HPO4, 10 mM NaF, 2 mM EDTA, 34 μg/ml phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotonin, 1.5 mg/ml benzamide, 5 μg/ml leupeptin, 10 μg/ml antipain, 0.5 μg/ml pepstatin, and 5 μg/ml leupeptin. Muscle was washed, minced, and homogenized by aspiration about five times with a 15-gauge needle on a 1-ml syringe (Becton-Dickinson, Franklin Lakes, NJ). The lysates were centrifuged at 23,000 g for 50 min at 4°C.

Protein was determined using a Bio-Rad DC protein assay kit (Hercules, CA). Equal protein concentrations of clarified lysates (1 mg) were immunoprecipitated with anti-IRS-1 antibody, JD66 (the generous gift of Dr. Morris White, Joslin Diabetes Center, Boston, MA), overnight at 4°C. Antibody-antigen complexes were incubated for 2 h at 4°C with protein-A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). The beads were then washed three times with PBS (pH 7.4) containing 1% NP-40, two times with 100 mM Tris-HCl (pH 7.6) containing 500 mM LiCl, once with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 1 mM EDTA, and, finally, once in PI 3K assay buffer (20 mM Tris, pH 7.4, containing 100 mM NaCl, 10 mM MgCl2, 0.5 mM EGTA, and 120 μM adenosine). Washed beads were preincubated for 10 min at 30°C in 40 μl of PI 3K assay buffer and 5 μl of lipid mixture containing phosphatidylinerine and phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) in 20 mM HEPES, 1 mM EDTA (pH 7.4). Kinase reactions were started by the addition of 100 μM
ATP containing 15 μCi [32P]ATP (ICN, Costa Mesa, CA). After 10 min at 30°C, reactions were terminated by the addition of 1 N HCl, and phosphorylated lipids were extracted twice in chloroform-methanol (1:1). The lower organic phase containing the reaction products was spotted (15 μl) onto CDTA-treated silica gel 60 TLC plates and was resolved by the chromatography system of Walsh et al. (37). Labeled phosphatidylinositol 3-phosphate that comigrated with a phosphatidylinositol 4-phosphate standard was detected by autoradiography, scraped from the TLC plates, and quantified by Cerenkov scintillation counting. Results were expressed as counts per minute of [32P] per milligram of protein.

Immunoblotting. Approximately 50 mg of frozen muscle were pulverized, homogenized with a Virtis homogenizer (Gardiner, NY) in ice-cold buffer containing 20 mM Tris (pH 7.5), 5 mM EDTA, 10 mM Na3P04, 100 mM NaF, 2 mM Na3VO4, 1% IGEPAL CA-630, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (1, 15, 17). Tissue lysates were solubilized with gentle mixing for 1 h at 4°C and centrifuged at 14,000 g for 15 min at 4°C. Protein was determined by the Bio-Rad DC protein assay. Rat skeletal muscle was used as an internal standard for IR, IRS-1, and p85 immunoblots, and mouse liver was used as an internal standard for IRS-2 immunoblots. The same internal standard was run in all immunoblots for each protein, and data are expressed as the percentage of internal standard.

Solubilized proteins (150 μg) were resolved by 6% (IRSs) or 7.5% (insulin receptor, p85 subunit of PI 3K) SDS-PAGE and transferred to Immobilon P membrane (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dry milk or 7.5% (insulin receptor, p85 subunit of PI 3K) SDS-PAGE and transferred to Immobilon P membrane (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dry milk or BS A in 20 mM Tris (pH 7.5) containing 150 mM NaCl and 0.05% Tween-20 for 1 h at room temperature. The membrane was then incubated with 3% milk or BSA for 1 h in Tris-buffered saline (TBS) containing 0.05% Tween-20 at room temperature with antibodies to the insulin receptor β-subunit (Transduction Laboratories, Lexington, KY), IRS-1 or IRS-2 (the generous gifts of Dr. Morris White), or p85 α-subunit of PI 3K (Upstate Biotechnology, Lake Placid, NY), followed by 1 h of incubation with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and washed with TBS containing 0.05% Tween-20. The bands were visualized using the Enhanced Chemiluminescence System (Amer sham, Arlington Heights, IL) and quantified by densitometer (Bio-Rad).

RNA isolation and RT-PCR. Total RNA was isolated from muscle biopsies (~30 mg) by use of the SV Total RNA isolation system (Promega, Madison, WI). The protocol provided by the manufacturer was followed. The RNA samples were kept in RNase free water at −80°C. RT-PCR was performed in 25 μl of the Access RT-PCR system from Promega supplemented with 1 μg of each sense and antisense primer. The primers for human IRS-1 mRNA were 5'-GGAGACGGAT-GGCTTCTCAGGAGCTG-3' and 5'-GGTTCAGGATACACTTG-GCCAGACCTCT-3'. The primers for human IRS-2 mRNA were 5'-AGATCGTGTCAGTTATACCAGG-3' and 5'-CTTACGATGCAGGAGTATATCAGG-3'. For human actin mRNA, the primers were 5'-GGCAGATGATCCGAGA-3' and 5'-CATGAGATCCGAGA-3'. The reaction mixture was incubated by 1 cycle of 45 min at 48°C and 2 min at 94°C, 40 cycles of 1 min at 62°C and 1 min at 68°C, and one cycle of 7 min at 68°C. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide staining. The intensity of the DNA bands was determined by a laser densitometer (Bio-Rad). The identity of the DNA fragments was confirmed by DNA sequencing.

Data analysis. IMGU was calculated as the mean glucose infusion rate per square meter per minute. IMGU at the time of each biopsy was used for statistical analyses, and IMGU every 5 min throughout the study is depicted graphically in Fig. 1. Because hepatic glucose production is not completely suppressed at the 40 mU·m−2·min−1 insulin dose in PCOS (11, 12), IMGU at this dose slightly underestimated actual insulin-mediated glucose disposal. However, we have demonstrated previously that IMGU corrected for residual hepatic glucose production (HGP) remains significantly decreased at the 40 mU·m−2·min−1 insulin dose in PCOS (11, 12). Therefore, we elected not to measure HGP in this study. Comparisons between control and PCOS women for clinical features and baseline biochemical parameters were made by unpaired t-tests. Repeated-measures ANOVA was used to determine the differences in responses to insulin in PCOS and in control women during the clamp studies. Pearson correlation coefficients were determined to assess whether there were any significant linear correlations between variables. Differences were considered to be significant at P ≤ 0.05. Data are reported as means ± SE. Statistical calculations were performed using the SAS program (SAS Institute, Cary, NC).

RESULTS

Clinical and Biochemical Features

PCOS and control women had comparable ages and body weights by design. PCOS women had significantly increased levels of total T and uT (both P < 0.001). DHEAS levels did not differ significantly in PCOS compared with control women. Fasting glucose levels did not differ significantly, whereas levels 2 h after a 75-g oral glucose load were significantly higher in PCOS compared with control women (P < 0.05), and three PCOS women had impaired glucose tolerance by World Health Organization criteria (30). Fasting insulin levels did not differ, but 2 h post-75-g glucose load insulin levels (P < 0.05) were significantly increased in PCOS compared with control women. The androgen, glucose, and insulin levels were all consistent with the
Table 1. Clinical and biochemical features

<table>
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<th>PCOS</th>
<th>Control</th>
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<tr>
<td>n</td>
<td>14</td>
<td>12</td>
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<tr>
<td>Age, yr</td>
<td>29 ± 1</td>
<td>30 ± 2</td>
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<tr>
<td>BMI, kg/m²</td>
<td>40.5 ± 1.9</td>
<td>40.5 ± 1.6</td>
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<tr>
<td>T, ng/dl</td>
<td>91 ± 7</td>
<td>33 ± 3**</td>
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<tr>
<td>uT, ng/dl</td>
<td>29 ± 1</td>
<td>9 ± 1**</td>
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<tr>
<td>DHEAS, µg/dl</td>
<td>207 ± 25</td>
<td>157 ± 15</td>
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<tr>
<td>Glucose, mg/dl</td>
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<td></td>
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<tr>
<td>0 h</td>
<td>86 ± 1</td>
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<td>2 h</td>
<td>128 ± 4</td>
<td>115 ± 5*</td>
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<td>Insulin, µU/ml</td>
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<tr>
<td>0 h</td>
<td>25 ± 3</td>
<td>18 ± 2</td>
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<td>2 h</td>
<td>187 ± 20</td>
<td>112 ± 26*</td>
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Values are means ± SE; n, no. of women/group. PCOS, polycystic ovary syndrome; BMI, body mass index; T, testosterone; uT, unbound testosterone; DHEAS, dehydroepiandrosterone sulfate; P*, P < 0.05; **P < 0.01 compared with respective PCOS group by unpaired t-test.

Table 1 describes the well-described biochemical profile of PCOS (9–13) (Table 1).

**Euglycemic Clamp Studies**

The 40 mU·m⁻²·min⁻¹ insulin dose achieved steady-state insulin levels of ~100 µU/ml and the 400 mU·m⁻²·min⁻¹ dose achieved steady-state insulin levels of ~2,000 µU/ml. IMGU throughout the study was significantly decreased in the PCOS compared with the control women (P < 0.05; Fig. 1), whereas steady-state insulin levels during the study did not differ significantly (data not shown). There were no significant differences in basal IRS-1-associated PI 3K activity (Fig. 2). IRS-1-associated PI 3K activity increased significantly from baseline at all biopsy time points of the insulin infusions in control women, except 90 min (Fig. 2). IRS-1-associated PI 3K activity increased significantly (data not shown). There were no significant differences in the abundance of insulin receptor substrate (IRS)-1- and IRS-2-associated PI 3K activity in PCOS compared with control women (167 ± 23% PCOS, n = 13 vs. 101 ± 11% control, n = 11, P < 0.05; Fig. 4). The abundance of IRS-2 was significantly increased in PCOS compared with control women (176 ± 23% PCOS, n = 13 vs. 101 ± 11% control, n = 11, P < 0.05; Fig. 4). Thus decreases in IRS-1-associated PI 3K activity could not be accounted for by a decrease in the amount of signaling proteins. Both IRS-1 and IRS-2 mRNAs were detected in the PCOS (n = 10) and control (n = 10) muscle samples. When normalized to actin mRNA, IRS-1 (0.47 ± 0.06 PCOS vs. 0.33 ± 0.03 control, P = NS) and IRS-2 (0.13 ± 0.02 PCOS vs. 0.17 ± 0.01 control, P = NS) mRNA expression was similar in PCOS and control skeletal muscle. These discrepant findings between immunoblot and RT-PCR results could reflect differences in protein compared with mRNA stability or the relative insensitivity of the RT-PCR technique (18).

**Pearson Correlations**

There were no significant correlations between body mass index (BMI), fasting glucose or insulin levels, IMGU, and basal or insulin-stimulated IRS-1-associated PI 3K activity. The abundance of signaling proteins was not significantly correlated with either basal or insulin-stimulated IRS-1-associated PI 3K activity or with BMI, fasting glucose or insulin levels, or IMGU.

There were no significant differences in the abundance of IR [50 ± 7% PCOS vs. 49 ± 7% control, P = nonsignificant (NS)], IRS-1 (41 ± 6% PCOS vs. 36 ± 5%, P = NS), or the p85 regulatory subunit of PI 3K (70 ± 6% PCOS vs. 66 ± 6% control, P = NS) in the baseline muscle biopsies from PCOS (n = 14) and control (n = 12) women (Fig. 4). The abundance of IRS-2 was significantly increased in PCOS compared with control women (167 ± 23% PCOS, n = 13 vs. 101 ± 11% control, n = 11, P < 0.05; Fig. 4). Thus decreases in IRS-1-associated PI 3K activity could not be accounted for by a decrease in the amount of signaling proteins. Both IRS-1 and IRS-2 mRNAs were detected in the PCOS (n = 10) and control (n = 10) muscle samples. When normalized to actin mRNA, IRS-1 (0.47 ± 0.06 PCOS vs. 0.33 ± 0.03 control, P = NS) and IRS-2 (0.13 ± 0.02 PCOS vs. 0.17 ± 0.01 control, P = NS) mRNA expression was similar in PCOS and control skeletal muscle. These discrepant findings between immunoblot and RT-PCR results could reflect differences in protein compared with mRNA stability or the relative insensitivity of the RT-PCR technique (18).

**Pearson Correlations**

There were no significant correlations between body mass index (BMI), fasting glucose or insulin levels, IMGU, and basal or insulin-stimulated IRS-1-associated PI 3K activity. The abundance of signaling proteins was not significantly correlated with either basal or insulin-stimulated IRS-1-associated PI 3K activity or with BMI, fasting glucose or insulin levels, or IMGU.

**Euglycemic Clamp Studies**

The 40 mU·m⁻²·min⁻¹ insulin dose achieved steady-state insulin levels of ~100 µU/ml and the 400 mU·m⁻²·min⁻¹ dose achieved steady-state insulin levels of ~2,000 µU/ml. IMGU throughout the study was significantly decreased in the PCOS compared with the control women (P < 0.05; Fig. 1), whereas steady-state insulin levels during the study did not differ significantly (data not shown). There were no significant differences in basal IRS-1-associated PI 3K activity (Fig. 2). IRS-1-associated PI 3K activity increased significantly from baseline at all biopsy time points of the insulin infusions in control women, except 90 min (P < 0.05–0.001; Figs. 2 and 3). In PCOS women, IRS-1-associated PI 3K activity did not differ significantly from baseline until the 400 mU·m⁻²·min⁻¹ insulin dose (P < 0.05–0.01; Figs. 2 and 3).

There were significant decreases in IRS-1-associated PI 3K activity in PCOS compared with control women (P < 0.05; Figs. 2 and 3). However, differences in degree of stimulation of IRS-1-associated PI 3K activity did not achieve statistical significance, most likely because baseline as well as insulin-stimulated PI 3K activity tended to be lower in PCOS. The maximal degree of stimulation of IRS-1-associated PI 3K activity was 3.5 ± 0.8-fold in PCOS and 4.7 ± 1.2-fold in control women. The +30-min PI 3K activity differed significantly at the 40 mU·m⁻²·min⁻¹ insulin dose (P < 0.05), and the +15-min PI 3K activity differed at the 400 mU·m⁻²·min⁻¹ dose (P = 0.09; Fig. 2). At the higher insulin dose, there was a decrease in PI 3K activity at +30 min after the +15-min peak in both groups. PI 3K activity increased by +90 min to 3.3 ± 0.6-fold in PCOS and 3.7 ± 0.5-fold in control women (Fig. 2). The maximal difference of ~50% in IRS-1-associated PI 3K activity in PCOS compared with control women was similar at both insulin doses (Fig. 2) and occurred in parallel with the maximal difference in glucose infusion rates of ~30% at the time of those biopsies (Fig. 1).

**Abundance of Signaling Proteins and IRS mRNA Expression**

There were no significant differences in the abundance of IR [50 ± 7% PCOS vs. 49 ± 7% control, P = nonsignificant (NS)], IRS-1 (41 ± 6% PCOS vs. 36 ± 5%, P = NS), or the p85 regulatory subunit of PI 3K (70 ± 6% PCOS vs. 66 ± 6% control, P = NS) in the baseline muscle biopsies from PCOS (n = 14) and control (n = 12) women (Fig. 4). The abundance of IRS-2 was significantly increased in PCOS compared with control women (167 ± 23% PCOS, n = 13 vs. 101 ± 11% control, n = 11, P < 0.05; Fig. 4). Thus decreases in IRS-1-associated PI 3K activity could not be accounted for by a decrease in the amount of signaling proteins. Both IRS-1 and IRS-2 mRNAs were detected in the PCOS (n = 10) and control (n = 10) muscle samples. When normalized to actin mRNA, IRS-1 (0.47 ± 0.06 PCOS vs. 0.33 ± 0.03 control, P = NS) and IRS-2 (0.13 ± 0.02 PCOS vs. 0.17 ± 0.01 control, P = NS) mRNA expression was similar in PCOS and control skeletal muscle. These discrepant findings between immunoblot and RT-PCR results could reflect differences in protein compared with mRNA stability or the relative insensitivity of the RT-PCR technique (18).
DISCUSSION

Muscle is responsible for ~85% of IMGU and is thus the most important insulin-resistant tissue on a quantitative basis (8). We have found a significant decrease in skeletal muscle insulin-mediated activation of IRS-1-associated PI 3K in parallel with a significant decrease in IMGU in PCOS. This finding was independent of obesity and type 2 DM (1, 6, 17, 25, 27). There were no significant differences in the abundance of the IR, IRS-1, or the p85 regulatory subunit of PI 3K. Accordingly, because the PI 3K activity measured was that associated with IRS-1, the differences in activity could not be accounted for by differences in the abundance of IRS-1 or PI 3K. This signaling defect could be partially reversed, because IRS-1-associated PI 3K activity was increased significantly above baseline at the 400 mU·m⁻²·min⁻¹ insulin dose in PCOS. Adipocytes isolated from PCOS women also have a postbinding defect in insulin signaling (5, 12), although PI 3K activation has not been explored in this cell type. IRS-2 was expressed in PCOS and control skeletal muscle, and the abundance of IRS-2 protein was increased in PCOS compared with control women, suggesting that this might reflect a compensatory change similar to that seen in mice with targeted disruption of the IRS-1 gene (35, 39) or after exercise in rats (4). The increase in IRS-1-associated PI 3K activity over baseline in PCOS women during the 40 mU·m⁻²·min⁻¹ insulin infusion did not achieve statistical significance, suggesting that IRS-2-associated PI 3K activation may have been the predominant pathway mediating IMGU analogous to adipocytes from individuals with type 2 DM (33). However, we had insufficient sample to measure IRS-2-associated PI 3K activation; hence, we could not address this hypothesis directly. Nevertheless, IMGU was decreased in PCOS, suggesting that
IRS-2-associated PI 3K activity could not completely compensate for defective IRS-1-mediated PI 3K activation. This is consistent with recent studies suggesting that IRS-2 does not play a major role in skeletal muscle IMGU in sedentary animals or after short-term exercise (21).

Significant differences in IRS-1-associated PI 3K activation became apparent only when we obtained biopsies at early time points after starting or increasing the insulin infusion. Studies in rodents and humans in vivo (19, 20) and in human skeletal muscle strips incubated in vitro (17, 27) have found a rapid peaking of IR and IRS-1 tyrosine phosphorylation and/or PI 3K activation followed by a decrease in these signaling events. We also noted a decrease in PI 3K activity after a rapid peak at higher insulin doses. These observations suggest that signaling events initiating glucose transport occur rapidly. A recent study (25) of in vivo IRS-1- and IRS-2-associated PI 3K activation at insulin doses of 120 and 300 mU·m⁻²·min⁻¹ reported that pilot studies did not demonstrate differences in PI 3K activation at 15 min compared with 3 h. However, a decrease in PI 3K activity could have escaped detection because 30-min biopsies were not obtained.

It is unlikely that the muscle biopsy techniques contributed to our findings, because separate sites for each biopsy were used in the study of Hickey et al. (20), who also noted a peaking of PI 3K activity followed by a decrease. Furthermore, the decline in PI 3K activity was not observed in another study (40) where two biopsies were taken from the same incision. The maximal degree of stimulation of PI 3K activity was greater in those studies (20, 40) than in ours. However, those subjects were lean and insulin sensitive. The maximal degree of stimulation in our control women was similar to that seen in the obese control subjects from other studies at comparable insulin doses with the same (1) or separate (25) biopsy sites. This also provides further support for the conclusion that taking multiple biopsies from one incision did not affect our results. IMGU has been reported to be significantly decreased in obese control compared with lean control subjects without significant changes in IRS-1- or IRS-2-associated PI 3K activity (6, 25). This suggests that relatively modest changes in IR signaling can be associated with more marked differences in downstream insulin effects. It is also possible that signaling over time was decreased, similar to our findings, and that this may have escaped detection because serial samples were not examined in those studies (6, 25).

In morbidly obese subjects, insulin-stimulated IRS-1-associated PI 3K activation was decreased in skeletal muscle strips compared with lean individuals (17). A decrease in the tyrosine phosphorylation of the IR, IRS-1, and the p85 subunit of PI 3K, as well as a decreased abundance of these signaling proteins, was also present in these obese subjects (17). In type 2 DM, decreased IRS-1- and IRS-2-associated PI 3K activation has been reported without changes in the abundance of these signaling molecules (1, 6, 25, 27). In gestational DM (GDM), IR and IRS-1 phosphorylation were decreased with decreased IRS-1 abundance, whereas IRS-2 and p85 abundance were increased (15). The signaling defects could not be accounted for by obesity per se, because the decreases were significantly greater than those seen in weight-matched control subjects in PCOS, GDM, and type 2 DM (1, 15, 27). All of these conditions are characterized by decreased IMGU and hyperinsulinemia; hyperglycemia is present in type 2 and GDM (1, 6, 11, 12, 15, 17, 25, 27). Thus obesity, hyperglycemia, and hyperinsulinemia could not account for the observed differences in the abundance of signaling proteins in PCOS compared with type 2 or GDM (1, 15, 27). This is supported by our failure to find significant correlations between these parameters and signaling protein abundance.

There were several paradoxical observations in the present as well as in our previous studies with respect to insulin-signaling defects in PCOS. First, we found no decrease in IRS-1-associated PI 3K in PCOS-cultured fibroblasts, despite significant resistance to insulin-stimulated glycogen synthesis and documented abnormalities in IR phosphorylation in these cells (2, 13). In the present study, we found significant decreases in IRS-1-associated PI 3K in PCOS skeletal muscle. Tissue differences in insulin signaling may have accounted for these discrepant findings (24, 32). Second, in our original report, only ~50% of PCOS women had marked constitutive increases in IR phosphoserine content, but PCOS women without this defect had a similar decrease in insulin sensitivity. In the present study, IRS-1-associated PI 3K activation was consistently decreased in PCOS skeletal muscle, rather than in ~50% of PCOS who would be predicted to have increased IR serine phosphorylation. Thus there may be other signaling defects producing insulin resistance in those subjects without putative abnormalities in IR phosphorylation. It is possible that factors modulating IR tyrosine kinase activity or IRS-1 phosphorylation, such as plasma cell differentiation factor-1 (29) or tumor necrosis factor-α (22), contribute to insulin resistance in PCOS. Alternatively, the same factor that serine-phosphorylates the IR may also serine-phosphorylate downstream molecules, such as IRS-1, and inhibit signaling. Isoforms of protein kinase C are candidate kinases that can serine-phosphorylate both the IR and IRS-1 (7, 38). We had insufficient tissue to examine serine phosphorylation; hence, we could not directly investigate this hypothesis.

In summary, there was decreased IRS-1-associated PI 3K activation in association with decreased IMGU in PCOS. There were no significant differences in the abundance of the IR, IRS-1, or the p85 subunit of PI 3K consistent with a defect in signaling. IRS-2 abundance was significantly increased, suggesting a compensatory change, but this did not restore normal IMGU. Insulin signaling events occurred rapidly and escaped detection when biopsies were not obtained at early time points during insulin administration.

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