Impaired Insulin-Stimulated Phosphorylation of Akt and AS160 in Skeletal Muscle of Women With Polycystic Ovary Syndrome Is Reversed by Pioglitazone Treatment

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OBJECTIVE—Insulin resistance in skeletal muscle is a major risk factor for type 2 diabetes in women with polycystic ovary syndrome (PCOS). However, the molecular mechanisms underlying skeletal muscle insulin resistance and the insulin-sensitizing effect of thiazolidinediones in PCOS in vivo are less well characterized.

RESEARCH DESIGN AND METHODS—We determined molecular mediators of insulin signaling to glucose transport in skeletal muscle biopsies of 24 PCOS patients and 14 matched control subjects metabolically characterized by euglycemic-hyperinsulinemic clamps and indirect calorimetry, and we examined the effect of 16 weeks of treatment with pioglitazone in PCOS patients.

RESULTS—Impaired insulin-mediated total (R_d) oxidative and nonoxidative glucose disposal (NOGD) was paralleled by reduced insulin-stimulated Akt phosphorylation at Ser473 and Thr308 and AS160 phosphorylation in muscle of PCOS patients. Akt phosphorylation at Ser473 and Thr308 correlated positively with R_d and NOGD in the insulin-stimulated state. Serum free testosterone was inversely related to insulin-stimulated R_d and NOGD in PCOS. Importantly, the pioglitazone-mediated improvement in insulin-stimulated glucose metabolism, which did not fully reach normal levels, was accompanied by normalization of insulin-mediated Akt phosphorylation at Ser473 and Thr308 and AS160 phosphorylation. AMPK activity and phosphorylation were similar in the two groups and did not respond to pioglitazone in PCOS patients.

CONCLUSIONS—Impaired insulin signaling through Akt and AS160 in part explains insulin resistance at the molecular level in skeletal muscle in PCOS, and the ability of pioglitazone to enhance insulin sensitivity involves improved signaling through Akt and AS160. Moreover, our data provide correlative evidence that hyperandrogenism in PCOS may contribute to insulin resistance. ***Diabetes*** **57:357–366, 2008**

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AMPK, AMP-activated protein kinase;AS160, Akt substrate of 160 kDa;FDR, first-degree relative; GAP, GTPase-activating protein;IR, insulin receptor; IRS-1, insulin receptor substrate-1; NOGD, nonoxidative glucose disposal; PCOS, polycystic ovary syndrome; PI3K, phosphatidylinositol-3 kinase; PPAR, peroxisome proliferator–activated receptor; TZD, thiazolidinedione.

Polycystic ovary syndrome (PCOS) is a common endocrine disorder of unknown etiology characterized by hyperandrogenism, anovulatory infertility, and, frequently, profound insulin resistance in premenopausal women (1). Skeletal muscle is the major site of insulin-stimulated glucose disposal, and insulin resistance in this tissue represents a major risk factor for type 2 diabetes in women with PCOS (1,2). The molecular mechanisms underlying skeletal muscle insulin resistance in PCOS in vivo are less well characterized, but appear to involve impaired insulin-mediated association of phosphatidylinositol-3 kinase (PI3K) with insulin receptor substrate-1 (IRS-1) and enhanced serine (Ser) phosphorylation of the insulin receptor (IR) and IRS-1 (3–6). To what extent these abnormalities affect downstream insulin signaling to glucose transport in PCOS is at present unknown.

Experimental studies have provided evidence that insulin stimulation of GLUT4 translocation is dependent on phosphorylation of the Akt substrate of 160 kDa (AS160) (7–9). AS160 contains a Rab GTPase-activating protein (GAP) domain, the activity of which under basal conditions is sufficient to inhibit a Rab protein required for GLUT4 translocation. Upon insulin stimulation, phosphorylation of AS160 by Akt suppresses its GAP activity to a degree that permits exocytosis of GLUT4 vesicles to the plasma membrane. Recently, AMP-activated protein kinase (AMPK) was identified as a potential upstream kinase for AS160 in skeletal muscle, suggesting that AS160 may be a convergent point for different stimuli regulating GLUT4 translocation and glucose transport (10–12). Impaired insulin-mediated phosphorylation of Akt Thr308 and AS160 has been reported in skeletal muscle of nonobese type 2 diabetic patients (13). Most studies, however, have failed to demonstrate impaired insulin action at the level of Akt in muscle of type 2 diabetic patients and their first-degree relatives (FDRs) (14–20). Similarly, no studies have shown abnormal muscle AMPK activity under basal conditions in type 2 diabetic patients compared with weight-matched control subjects (21–23). Nevertheless, abnormalities in AMPK, Akt, or AS160 in muscle could contribute to insulin resistance in women with PCOS.

Treatment of PCOS with thiazolidinediones (TZDs) improves peripheral insulin sensitivity and ovulation rates (2,24). The insulin-sensitizing effect of TZD is in part mediated by activation of peroxisome proliferator–activated receptor (PPAR)-γ, which is highly abundant in adipose tissue (25), and may involve increased adiponectin secretion from adipocytes (26). Thus, recombinant adiponectin stimulates fatty acid oxidation and glucose

TABLE 1
Clinical and metabolic characteristics of PCOS patients and control subjects

	Control subjects	PCOS patients	PCOS pretreatment	PCOS posttreatment
n	14	24	10	10
Age (years)	33.8 ± 2.1	31.6 ± 1.3	30.3 ± 2.1	
Weight (kg)	98.2 ± 3.8	96.2 ± 2.2	96.4 ± 2.5	95.5 ± 2.8
BMI (kg/m^2)	33.7 ± 1.7	33.3 ± 0.9	33.2 ± 0.9	33.0 ± 1.1
Body fat (%)	40.5 ± 1.6	40.3 ± 1.1	39.1 ± 1.3	39.8 ± 1.4
Plasma triglycerides (mmol/l)	0.86 ± 0.11	1.66 ± 0.18*	1.43 ± 0.22	1.15 ± 0.16
Serum free testosterone (mg/l)	0.025 ± 0.003	0.048 ± 0.005*	0.053 ± 0.009	0.048 ± 0.007
Plasma glucose (mmol/l)	5.6 ± 0.1	5.9 ± 0.1	5.9 ± 0.2	5.6 ± 0.1
Serum insulin (pmol/l)	51 ± 6	104 ± 12**	125 ± 22	69 ± 11††
Plasma FFA basal (mmol/l)	0.47 ± 0.04	0.44 ± 0.03	0.45 ± 0.05	0.41 ± 0.05
R_d basal (mg · min^{-1} · m^{-2})	72 ± 3	77 ± 2	75 ± 3	76 ± 4
R_d clamp (mg · min^{-1} · m^{-2})	297 ± 23	150 ± 9*	138 ± 18	188 ± 25†
Glucose oxidation basal (mg · min^{-1} · m^{-2})	52 ± 8	42 ± 3	46 ± 5	43 ± 10
Glucose oxidation clamp (mg · min^{-1} · m^{-2})	141 ± 17	86 ± 5*	80 ± 10	101 ± 12††
Lipid oxidation basal (mg · min^{-1} · m^{-2})	33 ± 3	39 ± 1	38 ± 1	40 ± 4
Lipid oxidation clamp (mg · min^{-1} · m^{-2})	1 ± 6	23 ± 2*	24 ± 4	16 ± 5
NOGD basal (mg · min^{-1} · m^{-2})	20 ± 7	35 ± 3	30 ± 5	34 ± 9
NOGD clamp (mg · min^{-1} · m^{-2})	157 ± 22	65 ± 6*	58 ± 12	87 ± 14†

Data are means ± SEM. Differences between control subjects and all PCOS patients before randomization to TZD as well as the effect of 16 weeks of treatment with 30 mg pioglitazone once daily in 10 PCOS patients were tested using one-way or two-way ANOVA for repeated measures. The 38 study subjects represent a subcohort of a total of 44 subjects for which data have been presented previously (2). *$P < 0.001$ and **$P < 0.01$ vs. PCOS patients; †$P < 0.01$ and ††$P < 0.05$ vs. pretreatment.

transport by activation of AMPK in rodent muscle (26). Treatment with rosiglitazone increased muscle AMPK activity in insulin-resistant rats (27) and type 2 diabetic patients (28), but the role of plasma adiponectin was not examined. Other studies of human skeletal muscle have indicated that the insulin-sensitizing effect of adiponectin is in part exerted by improved insulin signaling (29,30). Accordingly, treatment with different TZDs improved insulin action on IRS-1 tyrosine phosphorylation, IRS-1–associated PI3K activity, and Akt activity/phosphorylation in muscle of type 2 diabetic patients and their FDRs (31–33). Whether pioglitazone improves insulin sensitivity in PCOS by affecting either the AMPK pathway or phosphorylation of Akt and AS160 remains to be elucidated.

The aim of the present study was to investigate the molecular mechanisms of insulin resistance in skeletal muscle of women with PCOS, and the mechanisms by which treatment with pioglitazone improves insulin sensitivity. We studied AMPK and Akt, two major regulators of glucose transport, likely through AS160, in skeletal muscle of women with PCOS and well-matched healthy control subjects.

RESEARCH DESIGN AND METHODS

Subjects and design. Twenty-four obese women of fertile age with PCOS and 14 healthy women, matched according to age and BMI, participated in the study (Table 1). This cohort represents all the subjects from whom skeletal muscle biopsies were obtained during a euglycemic-hyperinsulinemic clamp before PCOS patients were randomized in a double-blind manner to 16 weeks of treatment with either 30 mg pioglitazone or placebo once daily, as reported previously (2). This dose of pioglitazone is known to induce clinically relevant effects without exposing the PCOS patients to an unnecessary high risk of side effects. In addition to these pretreatment biopsies, another set of muscle biopsies was obtained from 10 of the pioglitazone-treated PCOS posttreatment. None of these patients experienced side effects related to pioglitazone treatment (2). No effect on insulin-stimulated glucose metabolism or any other parameters was observed in the placebo group (2), and therefore the effect of placebo on muscle enzymes was not studied. Two PCOS patients had impaired fasting glucose, but all had A1C within the normal range. Control subjects had normal glucose tolerance, no family history of diabetes, and regular menses. None of the participants were taking medication known to affect hormonal or metabolic parameters. Informed consent was obtained from all subjects before participation. The study was approved by the local ethics committee and the Danish Medicines Agency and was performed in accordance with the Helsinki Declaration II. The trial is registered at www.clinicaltrials.gov (NCT00145340).

The euglycemic-hyperinsulinemic clamp studies were performed after an overnight fast as described (2). In brief, a 2-h basal tracer equilibration period was followed by infusion of insulin at a rate of 40 mU · m^{-2} · min^{-1} for 3 h. This rate of insulin infusion was chosen to study insulin sensitivity and insulin signaling in skeletal muscle during prandial-like physiological hyperinsulinemia. The studies were combined with indirect calorimetry, and rates of total glucose disposal (R_d), glucose and lipid oxidation, and nonoxidative glucose disposal (NOGD) were calculated as described (2). Muscle biopsies were obtained from the vastus lateralis muscle immediately before and after the 3-h insulin infusion period using a modified Bergström needle with suction under local anesthesia. Muscle samples were immediately blotted free of blood, fat, and connective tissue and frozen in liquid nitrogen within 30 s. Serum levels of insulin, free testosterone, and plasma glucose, triglyceride, and free fatty acids (FFAs) were assayed as described (2). Plasma adiponectin was analyzed as described by Frystyk et al. (34). Percent body fat was determined by the bioimpedance method.

Muscle homogenate preparation. Lysates and homogenates were prepared from 70 mg (wet wt) muscle, which was freeze-dried; dissected free of visible fat, blood, and connective tissue; and homogenized as described previously (35) Homogenates rotated end-over-end at 4°C for 1 h. Lysates were prepared from the homogenates by centrifuging 25 min at 17,500*g* and 4°C. Total protein content was analyzed by the bicinchoninic acid method (Pierce, Rockford, IL). Unless stated specifically, all chemicals were of analytic grade from Sigma-Aldrich (Denmark).

Total crude membranes. For determination of total GLUT4 content, total crude membranes were obtained from 30 mg (wet wt) muscle homogenized in sucrose buffer (250 mmol/l sucrose, 30 mmol/l HEPES, 2 mmol/l EGTA, 40 mmol/l NaCl, 2 mmol/l phenyl-methylsulfonyl fluoride [PMSF], pH 7.4). The homogenates were cleared by centrifugation at 1,000*g* for 5 min, and total crude membranes were obtained as the pellet after centrifugation at 190,000*g* (90 min, 4°C).

SDS-PAGE and Western blotting. Muscle lysate or homogenate proteins were separated using 5, 7.5, or 10% Tris-HCl gels (Biorad, Denmark) and were transferred (semi-dry) to polyvinylidene difluoride (PVDF) membranes (Immobilion Transfer Membrane; Millipore A/S, Denmark). Standard Western blotting procedures were used for detecting specific proteins as described previously (35). Following detection and quantification using a charge-coupled device (CCD) image sensor and 1D software (Kodak Image Station, 2000MM; Kodak, Denmark), protein content and phosphorylation level were expressed in arbitrary units relative to a standard curve obtained by loading a human skeletal muscle control sample in various amounts on each separate gel.

Antibodies used for Western Blotting. AMPK subunit isoforms α1, α2, and γ3 were as described previously (23); α-AMPK Thr172 and AS160 (PAS) phosphorylation (nos. 2531 and 9611; Cell Signaling Technology, Beverly, MA); AS160 protein (no. Ab24469 Abcam plc; Cambridge, U.K.); GLUT4 (no. AB1346, Chemicon, Temecula, CA); Akt1/2 protein (no. 06-558 Upstate Biotechnology, Waltham, MA). Secondary antibodies used were horseradish peroxidase–conjugated antibodies (P0448, P0447, and P0163) from DAKO, Denmark.

IRS-1–associated PI3K activity. IRS-1–associated PI3K activity was measured in IRS-1 immunoprecipitated from muscle lysates (400 μg protein) using an anti–IRS-1 antibody raised against the COOH terminus of IRS-1 provided by Dr. K. Siddle (Cambridge University, U.K.) (36). PI3K assay (30°C for 20 min) was performed as described previously (37).

Microtiter plate assay for measuring Akt phosphorylation. Phosphorylation of Akt on Ser473 and Thr308 was measured by a microtiter-based assay according to the procedure described by the manufacturer (Biosource Europa, Belgium). For the Akt Ser473 and Thr308 assays, 120 and 200 μg of homogenate protein, respectively, was used for capturing Akt protein. Tests were performed on both recombinant Akt protein and human muscle samples to ensure that the signal obtained was within the linear range of the assay.

AMPK activity. Isoform-specific AMPK activity was measured in the presence of 200 μmol/l AMP in immunoprecipitations from 300 μg of muscle lysate protein using the anti-α1 and -α2 AMPK antibodies and the AMARA-peptide (HAMARAASAAAIARRR; 100 μmol/l) as substrate as previously described (35).

Statistical analysis. Data calculation and statistical analysis were performed using the SSSP for Windows Version 10.0 program. Variables with skewed distribution (insulin, triglycerides, and free testosterone) were logarithmically transformed before statistical analyses. Results are given as means ± SEM. Statistical evaluation was performed by one- or two-way ANOVA with or without repeated measurements using Tukey's post-hoc testing. The relationships between continuous variables were examined by calculation of Pearson's correlation coefficients. Differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

Clinical and metabolic characteristics. As previously reported for the entire cohort ($n = 30$) (2), the PCOS patients in the present study ($n = 24$) had increased fasting levels of serum insulin, free testosterone, and plasma triglycerides (Table 1). Insulin-stimulated R_d was 50% lower in PCOS patients than in control subjects, and this was primarily accounted for by a 60% reduction in NOGD, but also a 39% decrease in glucose oxidation. Moreover, the ability of insulin to suppress lipid oxidation was impaired in PCOS patients. Treatment of PCOS subjects with pioglitazone significantly reduced fasting serum insulin (45%), and improved insulin-stimulated R_d (36%), glucose oxidation (26%), and NOGD (50%) (Table 1).

GLUT4 protein and IRS-1–associated PI3K activity. The content of GLUT4 protein in crude membrane extracts of skeletal muscle was similar in PCOS patients and control subjects (Fig. 1*A*). In addition, GLUT4 content did not change in response to insulin in any groups or by pioglitazone treatment in PCOS patients. Thus, impaired insulin action in the PCOS patients and the improved glucose metabolism achieved by pioglitazone were not due to changes in GLUT4 protein content. However, this does not exclude impaired insulin-mediated GLUT4 translocation or improvement in this in response to pioglitazone in PCOS. Insulin increased IRS-1-associated PI3K activity significantly in both groups, and there was no difference in either the basal or the insulin-stimulated state (Fig. 1*B*). In the subgroup of pioglitazone-treated PCOS patients, the insulin-mediated increase in IRS-1–associated PI3K activity was not significant before, but only after, pioglitazone treatment.

Akt signaling. Total Akt1/2 protein expression in skeletal muscle did not differ between the groups, and it was not affected by either insulin or pioglitazone (Fig. 2*A*). Basal

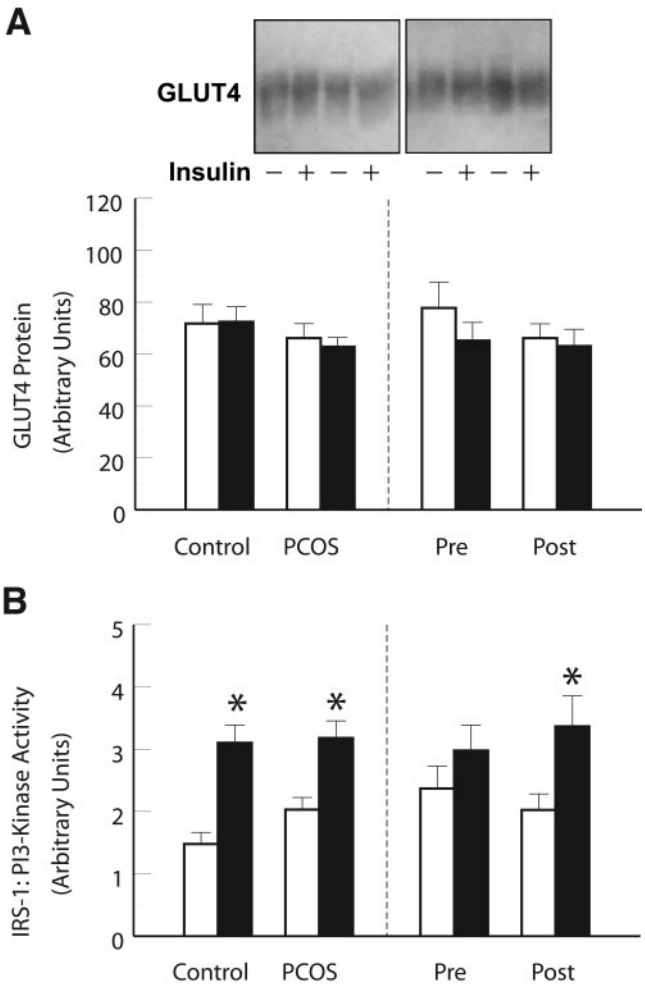

FIG. 1. GLUT4 protein content, representative immunoblot (*A*), and IRS-1–associated PI 3-kinase activity (*B*) in 14 control subjects and 24 PCOS patients and (right to the dotted line) in 10 PCOS patients before (Pre) and after (Post) 16 weeks of treatment with pioglitazone. Measurements were performed in skeletal muscle biopsies obtained during the basal (□) and insulin-stimulated (■) steady-state periods of a 3-h euglycemic-hyperinsulinemic clamp. Data are means ± SEM. *$P <$ 0.01 vs. corresponding basal values.

Akt phosphorylation at Ser473 and Thr308 was similar in skeletal muscle of PCOS patients and control subjects (Fig. 2*B* and *C*). Despite a significant increase in Akt phosphorylation at Ser473 and Thr308 in response to insulin in both groups, the effect of insulin on both sites was significantly attenuated (40–60%) in the PCOS patients. In the subgroup of PCOS patients undergoing pioglitazone treatment, insulin-mediated Akt phosphorylation at both Ser473 and Thr308 was restored to the levels observed in control subjects (Fig. 2*B* and *C*). Similar observations were done when data were adjusted for Akt protein, although the increase in insulin-mediated Ser473 phosphorylation did not reach statistical significance ($P =$ 0.17) (Fig. 2*D* and *E*).

AS160. The phosphorylation of AS160 was evaluated using an antibody recognizing proteins phosphorylated in the Akt recognition motif (R/K)X(R/K)XXS*/T*. A clear band at ~160 kDa was quantified, the identity of which was verified by an immuno-depletion experiment using an antibody recognizing AS160 independent of phosphorylation (Fig. 3*A*). AS160 protein content was not different between the two groups and did not change in response to

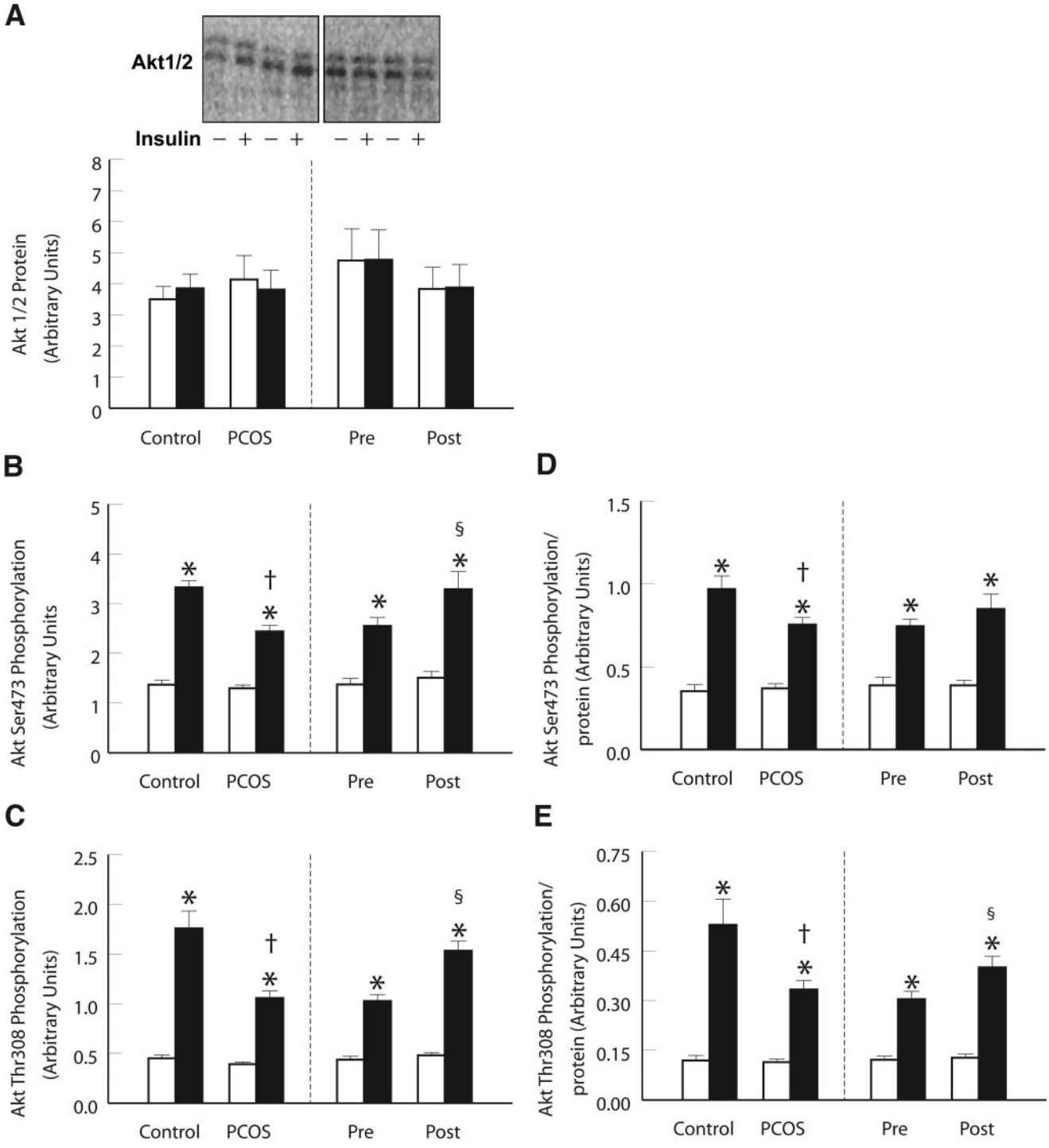

FIG. 2. Akt1/2 protein content and representative immunoblot (*A*), phosphorylation of Akt at Ser473 (*B*) and Thr308 (*C*), and phosphorylation of Akt at Ser473 (*D*) and Thr308 per protein (*E*) in 14 control subjects and 24 PCOS patients and (right to the dotted line) in 10 PCOS patients before (Pre) and after (Post) 16 weeks of treatment with pioglitazone. Measurements were performed in skeletal muscle biopsies obtained during the basal (□) and insulin-stimulated (■) steady-state periods of a 3-h euglycemic-hyperinsulinemic clamp. Data are means ± SEM. *$P < 0.01$ vs. corresponding basal values; †$P < 0.01$ vs. insulin-stimulated values in control subjects; §$P < 0.01$ vs. pretreatment insulin-stimulated values.

either insulin or treatment with pioglitazone, except for a small decrease in the pioglitazone-treated PCOS patients in the insulin-stimulated state (Fig. 3*B*). AS160 phosphorylation was increased significantly by insulin in both PCOS patients and control subjects (Fig. 3*C*). However, in accordance with the Akt phosphorylation data, insulin-stimulated AS160 phosphorylation tended ($P = 0.08$) to be reduced in the PCOS patients, and after pioglitazone treatment insulin-mediated AS160 phosphorylation tended ($P = 0.07$) to be restored to the level seen in the control subjects (Fig. 3*C*). When evaluating AS160 phosphorylation adjusted for AS160 protein content, these tendencies all became statistically significant ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 3*D*)

AMPK. Protein levels of the two catalytic (α1 and α2) subunits of AMPK were similar in PCOS and control

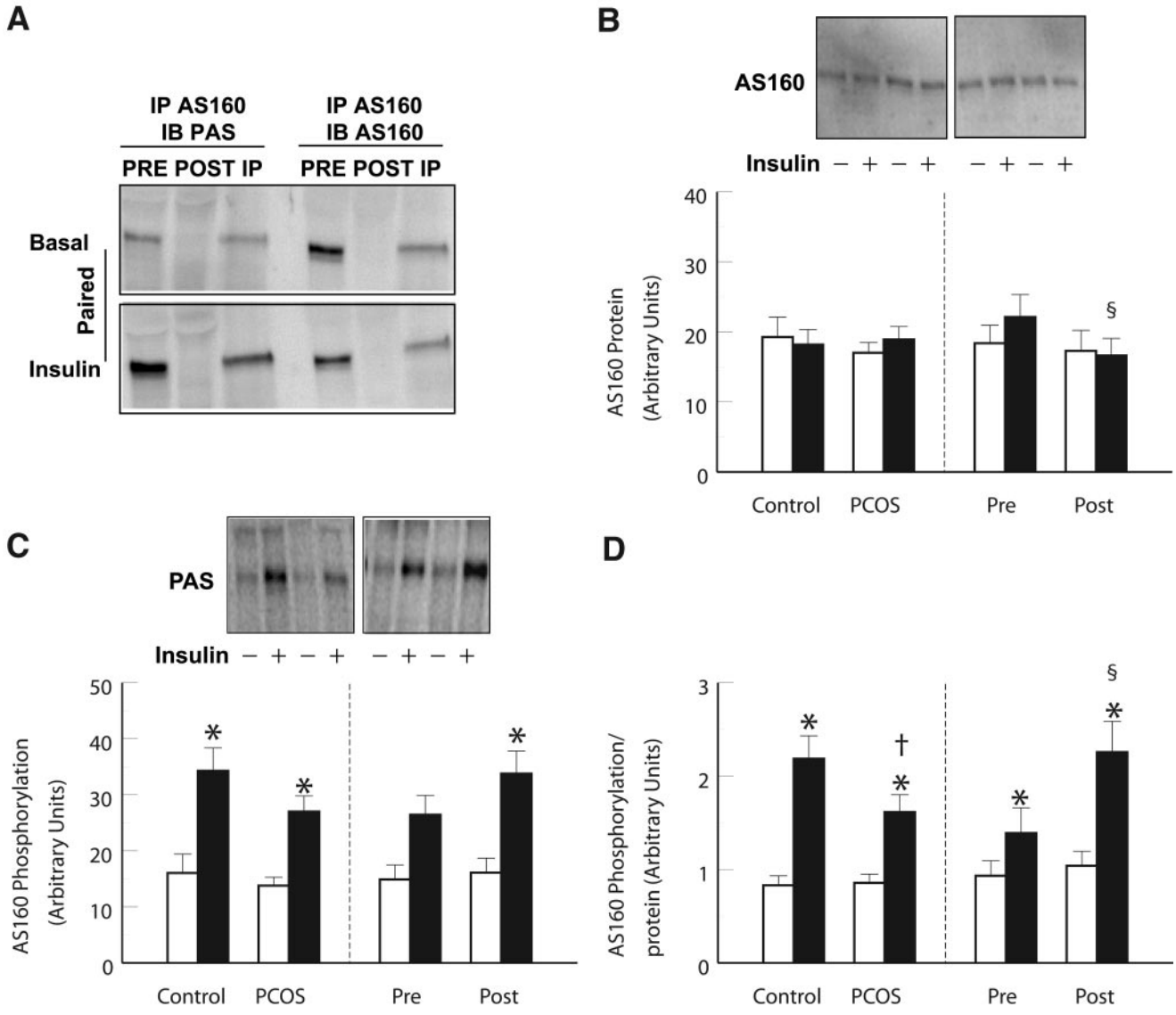

FIG. 3. *A*: **Immunoprecipitation (IP) of AS160 followed by immunoblotting (IB) using either anti–phospho-Akt substrate (PAS) (left) or anti AS160 antibodies (right) on nonstimulated (top) or insulin-stimulated (bottom) human muscle biopsies from a healthy individual. Samples of the incoming lysate (Pre), the remnant lysate after IP (Post), and the immunoprecipitate were loaded. AS160 protein content (*B*), AS160 phosphorylation (*C*), and AS160 phosphorylation per AS160 protein (*D*) in 14 control subjects and 24 PCOS patients and (right to the dotted line) in 10 PCOS patients before (Pre) and after (Post) 16 weeks of treatment with pioglitazone. Measurements were performed in skeletal muscle biopsies obtained during the basal (□) and insulin-stimulated (■) steady-state periods of a 3-h euglycemic-hyperinsulinemic clamp. Representative immunoblots are shown above *B* and *C*. Data are means ± SEM. **P* < 0.01 vs. corresponding basal values; †*P* < 0.01 vs. insulin-stimulated values in control subjects; §*P* < 0.05 vs. pretreatment insulin-stimulated values.**

subjects (Fig. 4*A*) and did not change in response to pioglitazone treatment (Fig. 4*B*). Surprisingly, protein content of the regulatory γ3 subunit of AMPK was increased in PCOS patients (Fig. 4*A*), and it remained elevated in the pioglitazone-treated subgroup of PCOS subjects (Fig. 4*B*). However, measurements of AMPK activity as either Thr172 phosphorylation on α-AMPK or as the activity associated with the α1 or α2 catalytic AMPK isoforms were not different in the PCOS patients compared with control subjects, and they did not change in response to insulin or pioglitazone treatment, except for a nonsignificant ($P = 0.054$) insulin-mediated decrease in α1-AMPK activity in control subjects (Fig. 5*A–C*). To examine whether the absent response was explained by failure of pioglitazone to increase adiponectin levels, we determined plasma adiponectin and observed a more than twofold increase in plasma adiponectin (6.8 ± 0.8 vs. 14.6 ± 2.1 mg/l; $P < 0.001$) after pioglitazone treatment in PCOS patients.

Akt signaling in relation to AS160 and glucose metabolism. In the total population, Akt phosphorylation at Ser473 and Thr308 correlated positively with AS160 phosphorylation during insulin stimulation (Fig. 6*A* and *B*). The association between Akt Ser473 and AS160 phosphorylation was significant in both PCOS ($r = 0.44$, $P = 0.04$) and control subjects ($r = 0.65$, $P = 0.02$), whereas the association between Akt Thr308 and AS160 phosphorylation was significant only in PCOS subjects ($r = 0.54$, $P = 0.009$). Insulin-stimulated Akt phosphorylation at Thr308 and Ser473 in skeletal muscle correlated strongly with insulin-stimulated R_d and NOGD (Fig. 6*C* and *F*). The association between insulin-stimulated R_d and AS160 phosphorylation did not reach statistical significance ($r = 0.30$, $P = 0.08$), but insulin-stimulated AS160 phosphorylation was posi-

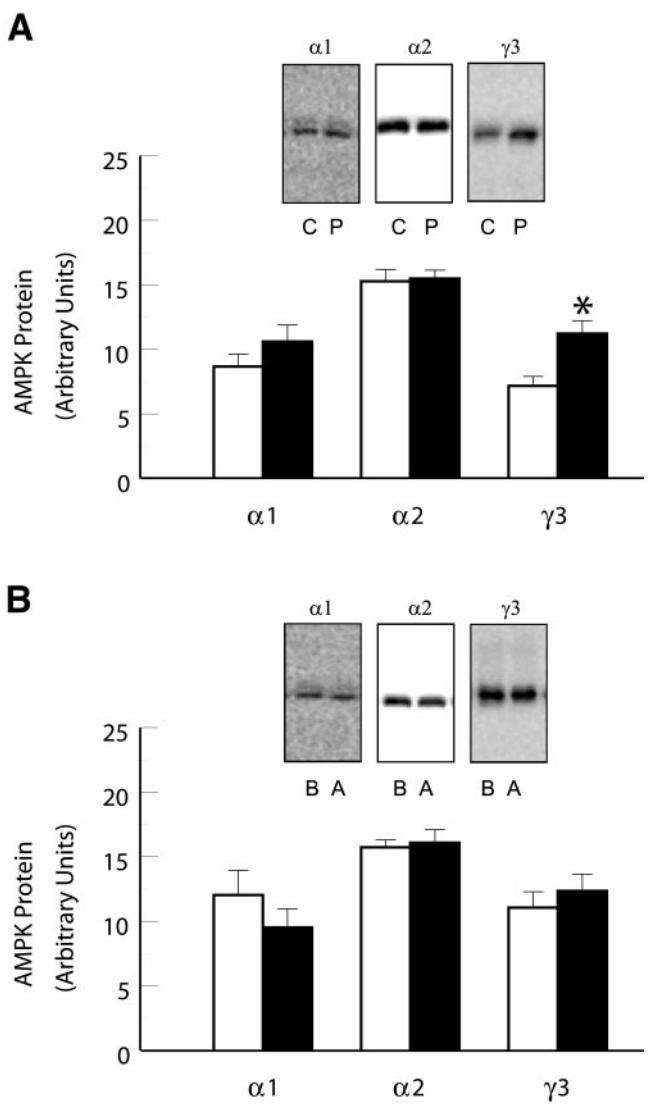

FIG. 4. Protein content of the α1, α2, and γ3 subunit of AMPK in skeletal muscle biopsies obtained under basal conditions in 14 control subjects (□) and 24 PCOS patients (■) (*A*) and in 10 PCOS patients before (□) and after (■) 16 weeks of treatment with pioglitazone (*B*). Representative immunoblots are shown above each graph. Data are means ± SEM. **P* < 0.01 vs. control subjects. C, control subjects; P, PCOS; B, before; A, after.

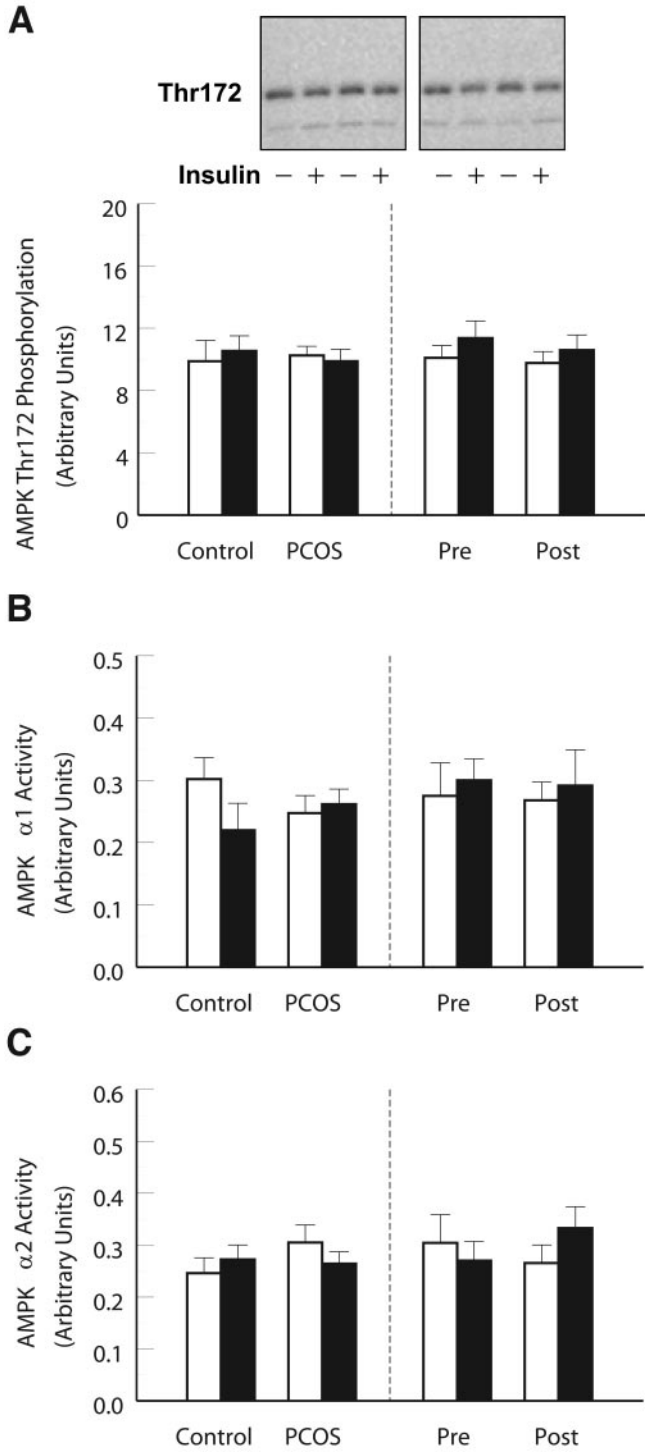

FIG. 5. AMPK Thr172 phosphorylation and representative immunoblot (*A*), AMPK α1 activity (*B*), and AMPK α2 activity (*C*) in 14 control subjects and 24 PCOS patients and (right to the dotted line) in 10 PCOS patients before (Pre) and after (Post) 16 weeks of treatment with pioglitazone. Measurements were performed in skeletal muscle biopsies obtained during the basal (□) and insulin-stimulated (■) steady-state periods of a 3-h euglycemic-hyperinsulinemic clamp. Data are means ± SEM.

tively associated with NOGD ($r = 0.42$, $P = 0.01$). In control subjects, the associations between Thr308 phosphorylation and R_d ($r = 0.81$, $P < 0.001$) and NOGD ($r = 0.76$, $P < 0.001$) were stronger than in PCOS patients ($r = 0.50$, $P = 0.01$, and $r = 0.56$, $P < 0.01$, respectively), and in control subjects only, Ser473 phosphorylation was positively associated with NOGD ($r = 0.59$, $P = 0.03$). No significant relationships between insulin-stimulated glucose metabolism and AS160 phosphorylation were observed in the individual groups.

In 8 of 10 PCOS patients, an increase in insulin stimulation of R_d and NOGM in response to pioglitzaone treatment was accompanied by an increase in phosphorylation of Akt at Ser473 and Thr308 ($P = 0.05$). However, there was no univariate correlation between the magnitude by which these measures of whole-body glucose disposal and phosphorylation of Akt increased (all $r < 0.25$).

Hyperandrogenism in insulin resistance. To explore the potential role of hyperandrogenism in insulin resistance, we examined the relationship between serum free testosterone and insulin-stimulated glucose metabolism and signaling through Akt and AS160. In the total population, serum free testosterone was negatively associated with insulin-stimulated R_d ($r = -0.47$, $P = 0.003$), NOGD ($r = -0.45$, $P = 0.005$), Akt phosphorylation at Ser473 ($r =$

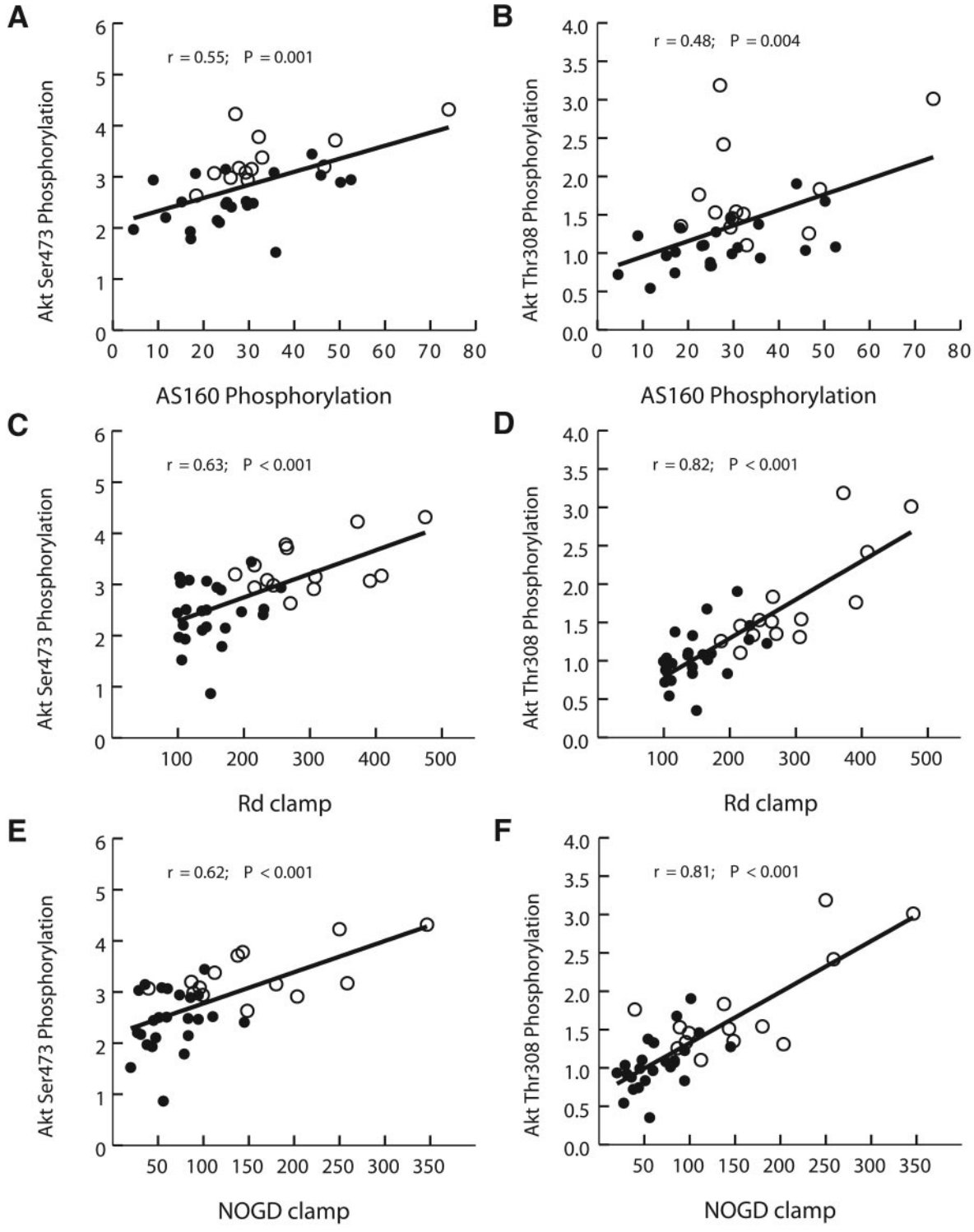

FIG. 6. The relationship of Akt Ser473 and Thr308 phosphorylation with AS160 phosphorylation in skeletal muscle (*A* and *B*) and rates (mg · m^{-2} · min^{-1}) of total glucose disposal (R_d clamp) (*C* and *D*) and nonoxidative glucose disposal (NOGD) (*E* and *F*) measured in the insulin-stimulated steady-state period of a 3-h euglycemic-hyperinsulinemic clamp in the total population of 14 control subjects (○) and 24 PCOS patients (●). Pearson's correlations coefficients for the total population are given.

-0.37, $P = 0.03$) and Thr308 ($r = -0.38$, $P = 0.02$), and AS160 phosphorylation ($r = -0.38$, $P = 0.03$). In PCOS patients, the association between free testosterone and insulin-stimulated R_d ($r = -0.42$, $P = 0.04$), and NOGD ($r = -0.47$, $P = 0.02$) remained significant, whereas no significant associations were found in control subjects.

DISCUSSION

In the present study, we investigated AMPK and insulin signaling to AS160 in skeletal muscle to define molecular mechanisms of insulin resistance in PCOS, as well as potential effects of 16 weeks of pioglitazone treatment on these signaling components. We provide evidence that

decreased insulin action on peripheral glucose metabolism is associated with impaired insulin signaling at the level of Akt and AS160 in women with PCOS. Moreover, we demonstrate that improved insulin signaling through Akt and AS160, in part, contributes to the insulin-sensitizing effect of pioglitazone treatment in PCOS. In contrast, AMPK activity was normal in skeletal muscle of women with PCOS, and it did not respond to pioglitazone despite a twofold increase in plasma adiponectin.

In cultured fibroblast from PCOS patients, impaired insulin action on glycogen synthesis was associated with increased basal IR Ser phosphorylation and decreased insulin-stimulated IR Tyr phosphorylation (3,38), whereas IRS-1–associated PI3K activity and mitogenic action of insulin were intact (38). In cultured human skeletal muscle cells, which express GLUT4, normal insulin-stimulated IR Tyr phosphorylation and IRS-1–associated PI3K activity were reported together with enhanced insulin action on glycogen synthesis and glucose transport in PCOS (4). In these myotubes, there was however increased basal IRS-1 Ser312 phosphorylation and enhanced mitogenic signaling through ERK1/2 (5). Studies of skeletal muscle in vivo have shown impaired insulin-stimulated IRS-1–associated PI3K activity after 30 min, but no difference after 90 min of physiological hyperinsulinemia in PCOS patients (6). In the present study, we found no abnormalities in IRS-1–associated PI3K activity in PCOS patients. We cannot exclude the possibility that we missed a transient lower IRS-1–associated PI3K activity. On the other hand, the PCOS and control subjects studied by Dunaif et al. (6) were morbidly obese, which may have contributed to a difference in IRS-1–associated PI3K activity. Studies of human skeletal muscle have demonstrated that insulin action on the proximal signaling components are sustained for several hours (14,37). For these reasons, it is unlikely that we missed any differences by taking the muscle biopsies after a 180-min insulin infusion. Thus, impaired insulin signaling through IRS-1 and PI3K in muscle does not seem to explain the reduction in insulin-stimulated glucose metabolism in obese PCOS patients.

Insulin signaling downstream of PI3K in skeletal muscle in PCOS has not been reported previously. Akt is an important mediator of insulin-stimulated GLUT4 translocation and glucose transport (39), and this process seems to be dependent on the phosphorylation of AS160 at several sites by Akt (7–9). The most important finding of the present study is a pronounced defect in insulin-mediated phosphorylation of Akt at Thr308 and Ser473 and of AS160 in muscle of PCOS patients. Most studies have failed to demonstrate abnormal Akt activity/phosphorylation in type 2 diabetic patients, FDRs, and diabetic myotubes (14–19,40). Recently, insulin-stimulated Akt and AS160 phosphorylation was found to be normal in muscle strips from FDRs, despite impaired glucose transport (20). However, reduced insulin action on Akt phosphorylation has been observed in FDRs with severe insulin resistance (41), in nonobese type 2 diabetic patients (13), and in subjects harboring an IR mutation (42). Thus, in certain insulin-resistant conditions, similar defects are seen and may even be of genetic origin. Although the defects at the level of AS160 and Akt in PCOS are seen before the development of type 2 diabetes and, hence, represent early abnormalities, further studies are needed to establish whether they are primarily of genetic or environmental origin.

We observed a strong positive relationship between R_d and NOGD and Akt phosphorylation during insulin stimulation, whereas AS160 phosphorylation showed less tight associations with Akt phosphorylation and insulin-stimulated glucose metabolism. This may reflect the possibility that AS160 is a substrate for multiple kinases (10–12). Nevertheless, these findings provide correlative evidence that Akt and AS160 phosphorylation are important mediators of insulin-stimulated glucose metabolism in skeletal muscle in vivo. In a study of nonobese type 2 diabetic patients, impaired insulin-mediated Akt Thr308 phosphorylation was associated with reduced AS160 phosphorylation in skeletal muscle (13). Thus, current available data indicate that impaired insulin-mediated Akt phosphorylation is paralleled by attenuated AS160 phosphorylation. In PCOS patients, reduced insulin action on Akt and AS160 in skeletal muscle seems to be independent of obesity and hyperglycemia. These findings may support the hypothesis of a unique sub-phenotype of skeletal muscle insulin resistance in PCOS (4).

The finding of impaired phosphorylation of Akt at both Ser473 and Thr308 despite normal insulin-stimulated PI3K activity incriminates modulators of Akt phosphorylation. Insulin resistance is strongly associated with increased lipid metabolites in human skeletal muscle, including ceramides (39). Ceramide-activated protein phosphatases, including protein phosphatase 2A (PP2A), have been shown to inhibit Akt by dephosphorylation (43). Increased ceramide levels were found together with impaired insulin-stimulated Akt Ser473 phosphorylation in muscle from obese subjects (44). Moreover, an impaired ability of insulin to suppress PP2A has been found in muscle of type 2 diabetic patients (45). Studies of intramyocellular lipid and ceramide content in PCOS are, however, unavailable, and a potential role for these in insulin resistance in PCOS therefore remains to be established.

The effect of TZD on insulin-signaling components in skeletal muscle of PCOS patients has not been studied previously. Here we report that pioglitazone improves insulin action on Akt and AS160. Treatment with pioglitazone also introduced a significant effect of insulin on PI3K activity in a subgroup of PCOS patients, but this effect was small and really there was no increase if compared with the total group of PCOS patients before treatment. Earlier studies have shown similar effects of TZD on insulin-stimulated PI3K activity and Akt phosphorylation in type 2 diabetic patients and FDRs (31–33), but a positive effect of TZD on AS160 phosphorylation has, to our knowledge, not been reported before. Intriguingly, a recent study concluded that the insulin-sensitizing effects of rosiglitazone are independent of enhanced insulin signaling through PI3K/Akt/AS160 in newly diagnosed type 2 diabetes (46). However, whether pretreatment levels of Akt and AS160 phosphorylation were reduced in these subjects was not reported. The finding that pioglitazone normalizes the most pronounced defects in insulin signaling may further support a potential unique role for impaired Akt and AS160 phosphorylation in skeletal muscle insulin resistance in PCOS. Whether the increase in AS160 and Akt phosphorylation is mediated by PPARγ-dependent or -independent effects of pioglitazone, and to what extent it involves the twofold increase in adiponectin, warrant further studies. There are data to support that high adiponectin may reduce IRS-1 Ser phosphorylation (47) and increase insulin-stimulated IR Tyr phosphorylation and glycogen synthase activity (29,30).

Defects in Akt and AS160 phosphorylation clearly rep-

resent pioglitazone-responsive markers of insulin resistance in PCOS, but the improvement of insulin-stimulated R_d and NOGD induced by pioglitazone only moved these measures about a third of the way toward where the control subjects were. This indicates that pioglitazone does not reverse all mechanisms underlying impaired insulin-stimulated glucose uptake in PCOS, and suggests that factors downstream of AS160 (e.g., Rab proteins), alternative Akt substrates, or even Akt-independent signals to GLUT4 translocation (48) could be impaired in PCOS. Factors outside the insulin-signaling cascade and muscle fibers (e.g., blood flow) could also be involved. The only factor, which can be suggested from the present study, is the levels of free androgens, which were higher in PCOS before treatment and were not normalized by pioglitazone. In support of this hypothesis, a negative association between androgen levels and insulin-stimulated glucose metabolism in women has been reported previously (49), and in the present study, free testosterone levels actually showed a closer inverse relationship with insulin-mediated R_d and NOGD than with Akt and AS160 phosphorylation in PCOS before treatment. However, further studies are needed to define the precise mechanisms by which androgens or other as yet unknown factors not improved by pioglitazone treatment impair insulin action on glucose metabolism.

Evaluation of AMPK was important for two reasons. First, AMPK was recently identified as an upstream kinase of AS160 (10–12). Second, a positive effect of chronic treatment with rosiglitazone on muscle AMPK activity has been reported in rodents (27) and type 2 diabetic patients (28). In both studies the effect was caused mainly by increased protein levels of AMPK. Although most studies of human skeletal muscle have been unable to detect abnormalities in measures of AMPK activity in type 2 diabetic patients and weight-matched control subjects (21–23), a recent study reported lower total AMPK activity in obese subjects with and without type 2 diabetes (28). We found, however, no difference in α2- or α1-AMPK protein content or activity, or Thr172 phosphorylation in PCOS patients, and no effect of prolonged pioglitazone treatment on these parameters. In PCOS subjects, there was a paradoxical increase in γ3 protein, which was unaffected by pioglitazone. The role of this increase, if any, remains to be determined. However, basal AS160 phosphorylation is preserved in γ3AMPK knockout mice (12). Overall, our data are consistent with normal basal AS160 phosphorylation in PCOS and no response to pioglitazone treatment. These data provide evidence that impaired AMPK activity plays no role for insulin resistance in PCOS, and that the insulin-sensitizing effect of pioglitazone in PCOS does not involve improved AMPK activity. Recombinant adiponectin stimulates AMPK leading to improved fatty acid oxidation and glucose transport in rodent muscle (26). However, we observed no effect of pioglitazone on AMPK despite a twofold increase in total adiponectin levels. Adiponectin is present in plasma as a trimer, hexamer, or high-molecular-weight (HMW) form (26), but as reported recently, only trimers of adiponectin seem to activate AMPK in muscle (50). However, other studies have shown that pioglitazone increases only the secretion of HMW adiponectin from adipocytes (51). Thus, a lack of increase in trimers of adiponectin could explain our findings.

In summary, the present study, to our knowledge for the first time, demonstrates impaired insulin signaling downstream of PI3K at the level of Akt and AS160 in skeletal muscle of women with PCOS. These molecular defects were fully reverted by prolonged treatment with pioglitazone in parallel with an improved but less-than-full reversal of insulin action on R_d and NOGD, and they in part explain the insulin-sensitizing effect of TZDs in PCOS. In contrast, muscle AMPK does not seem to play a role for neither insulin resistance nor the insulin-sensitizing effect of pioglitazone in PCOS. A role for hyperandrogenism in the lack of ability of pioglitazone to fully revert insulin-stimulated glucose metabolism warrants further studies.

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