

# Enhanced Mitogenic Signaling in Skeletal Muscle of Women With Polycystic Ovary Syndrome

Anne Corbould,<sup>1,2</sup> Haiyan Zhao,<sup>1</sup> Salida Mirzoeva,<sup>1</sup> Fraser Aird,<sup>1</sup> and Andrea Dunaif<sup>1,2</sup>

**Insulin resistance in polycystic ovary syndrome (PCOS) results from a postbinding defect in signaling. Insulin receptor and insulin receptor substrate (IRS)-1 serine hyperphosphorylation by an unidentified kinase(s) contributes to this defect. We investigated whether insulin resistance is selective, affecting metabolic but not mitogenic pathways, in skeletal muscle as it is in cultured skin fibroblasts in PCOS. Extracellular signal-regulated kinase (ERK)1/2 activation was increased in skeletal muscle tissue and in cultured myotubes basally and in response to insulin in women with PCOS compared with control women. Mitogen-activated/extracellular signal-regulated kinase kinase (MEK)1/2 was also activated in PCOS, whereas p38 mitogen-activated protein kinase phosphorylation and signaling from the insulin receptor to Grb2 was similar in both groups. The activity of p21Ras was decreased and Raf-1 abundance increased in PCOS, suggesting that altered mitogenic signaling began at this level. MEK1/2 inhibition reduced IRS-1 Ser<sup>312</sup> phosphorylation and increased IRS-1 association with the p85 subunit of phosphatidylinositol 3-kinase in both groups. We conclude that in PCOS skeletal muscle, 1) mitogenic signaling is enhanced in vivo and in culture, 2) ERK1/2 activation inhibits association of IRS-1 with p85 via IRS-1 Ser<sup>312</sup> phosphorylation, and 3) ERK1/2 activation may play a role in normal feedback of insulin signaling and contribute to resistance to insulin's metabolic actions in PCOS. *Diabetes* 55:751–759, 2006**

**P**olycystic ovary syndrome (PCOS), a common endocrinopathy characterized by disordered gonadotropin secretion and increased androgen production (1), is a leading risk factor for type 2 diabetes in premenopausal women (2,3). The disorder is associated with insulin resistance and pancreatic  $\beta$ -cell dysfunction. The molecular mechanisms of insulin resis-

tance in PCOS differ from those in other common conditions such as obesity and type 2 diabetes. There is a postbinding defect in insulin signaling in adipocytes (4,5) and skeletal muscle (6) acutely isolated from women with PCOS. In cultured skin fibroblasts of women with PCOS, impaired insulin action on glycogen synthesis is associated with constitutively increased insulin receptor  $\beta$ -subunit serine phosphorylation and decreased insulin receptor tyrosine kinase activity (7). A serine kinase extrinsic to the receptor is responsible for these abnormalities (8). In contrast to skin fibroblasts, the defects in glucose metabolism resolve in cultured skeletal muscle cells from women with PCOS (9), consistent with a major role of the in vivo environment in the pathogenesis of insulin resistance in this syndrome. Nevertheless, defects in insulin signaling persist in these cultured skeletal muscle cells, and phosphorylation of insulin receptor substrate (IRS)-1 on a key inhibitory site, Ser<sup>312</sup> (equivalent to Ser<sup>307</sup> in rat), is constitutively increased (9). These observations provide support for the hypothesis that enhanced serine phosphorylation of the insulin receptor and IRS-1 is an important mechanism for insulin resistance in PCOS.

Insulin has pleiotropic actions on cellular metabolism, growth, and differentiation (10,11). Insulin signaling pathways diverge upon activation of the insulin receptor. One pathway proceeds through IRS-1/2 and depends on activation of phosphatidylinositol (PI) 3-kinase to mediate insulin's actions on glucose metabolism, antilipolysis, and protein synthesis (10,11). Another pathway proceeds through binding of tyrosine-phosphorylated IRS-1/2 or Shc with Grb2/Sos, leading via p21Ras and Raf-1 to activation of the mitogen-activated protein kinase (MAPK) isoforms of extracellular signal-regulated kinase (ERK)1 and -2, thus mediating mitogenic and other gene-regulatory actions of insulin (10,11). ERK1 and -2 are members of a family of serine/threonine kinases, including p38 MAPK and c-Jun NH<sub>2</sub>-terminal kinase, that play important roles in cellular proliferation, differentiation, apoptosis, and also inflammation (12).

Insulin resistance in PCOS appears to be selective, affecting metabolic but not mitogenic actions of insulin in cultured skin fibroblasts (13). Selective impairment of metabolic insulin action has also been demonstrated in cultured skin fibroblasts (14) and skeletal muscle tissue (15,16) in type 2 diabetes. Whether insulin resistance in the skeletal muscle of women with PCOS is selective for metabolic pathways has not been examined. Selective insulin resistance may also be relevant to nonclassical insulin target tissues in PCOS. An intrinsic increase in the activity of multiple steroidogenic enzymes resulting in increased ovarian androgen production is a primary defect in PCOS (17). Selective insulin resistance may explain the

From the <sup>1</sup>Division of Endocrinology, Metabolism and Molecular Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois; and the <sup>2</sup>Division of Women's Health, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Andrea Dunaif, MD, Division of Endocrinology, Metabolism and Molecular Medicine, Feinberg School of Medicine, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611. E-mail: a-dunaif@northwestern.edu.

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A.C. and H.Z. contributed equally to this study.

AMPK, AMP-activated protein kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated/extracellular signal-regulated kinase kinase; PCOS, polycystic ovary syndrome; PI, phosphatidylinositol.

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apparent paradox of insulin acting through its cognate receptor to further stimulate ovarian androgen production in theca cells from women with PCOS (18), despite resistance to its effects on glucose metabolism.

We performed this study to determine whether the defects in skeletal muscle insulin action were selective for glucose metabolic pathways in PCOS. We found that the ERK1/2 pathway was constitutively activated both in vitro and in vivo in the skeletal muscle of women with PCOS. We examined the molecular mechanisms for the activation of this mitogenic/gene-regulatory pathway and also found evidence to suggest that ERK1/2 or ERK1/2-activated kinase(s) phosphorylate IRS-1 on Ser<sup>312</sup> and thus may contribute to the pathogenesis of skeletal muscle insulin resistance in PCOS.

## RESEARCH DESIGN AND METHODS

The study was approved by the institutional review boards of Brigham and Women's Hospital and Northwestern University. All subjects gave written informed consent. The subjects were aged 19–42 years, in good health, sedentary, and taking no medications known to affect carbohydrate or sex hormone metabolism for at least 1 month before the study, except for oral contraceptive agents, which were discontinued at least 3 months before study onset. Women with PCOS ( $n = 20$ ) had six or fewer menses per year and elevated total testosterone and/or non-sex hormone-binding globulin-bound testosterone levels (1). Control women ( $n = 15$ ) were of comparable age, weight, and ethnicity to PCOS subjects. They had menses every 27–35 days, no clinical or biochemical evidence of hyperandrogenism, normal glucose tolerance, and no first-degree relatives with diabetes. All subjects underwent a 75-g oral glucose tolerance test after an overnight fast and a 3-day, 300-g/day carbohydrate diet. Fasting and 2-h postchallenge glucose and insulin levels were measured, and World Health Organization criteria were used to assess glucose tolerance (19).

Skeletal muscle biopsies were obtained from vastus lateralis after an overnight fast as reported (6). Tissue from the first pass of the Bergstrom needle was immediately frozen for preparation of muscle lysates. Tissue from additional passes was used for cell culture. All tissue culture experiments were performed in pairs, i.e., cells from a PCOS subject were cultured simultaneously with those from a control subject.

**Cell culture.** Skeletal muscle was processed as reported (9,20). Experiments were performed on subcultured cells (passage 2) grown at a density of 3,000 cells/ml in skeletal muscle cell growth medium (Clonetics, BioWhittaker, Walkersville, MD) containing 2% fetal bovine serum (FBS; Life Technologies, Grand Island, NY) until near confluence, followed by 4 days in fusion medium ( $\alpha$ -minimum essential medium [Life Technologies] with 2% FBS, 50 IU/ml penicillin, and 50  $\mu$ g/ml streptomycin [Cellgro; Mediatech, Herndon, VA]). Differentiation, characterized by alignment of elongated myoblasts and fusion to form multinucleated myotubes, was monitored by phase-contrast microscopy. Before assays, the cultures were incubated in serum-free medium ( $\alpha$ -minimum essential medium with 0.1% BSA [fraction V; Boehringer Mannheim, Indianapolis, IN]) for time periods as indicated. The same lots of FBS and BSA were used for all experiments.

**Immunoprecipitation and immunoblotting.** Myotubes were incubated in serum-free medium for 2 h, followed by incubation in the same medium with or without insulin (100 nmol/l, 10 min) (Novo Nordisk, Princeton, NJ), and then scraped on ice in lysis buffer (6). Lysates were solubilized by rocking (40 min, 4°C) and then centrifuged for 10 min at 13,000g, and supernatants were stored at –80°C. Muscle biopsy samples were processed as described (6). Protein content was determined by DC protein assay (Bio-Rad Laboratories, Hercules CA). Lysates (50–100  $\mu$ g protein) were resolved by SDS-PAGE and immunoblotted with specific antibodies to ERK1/2, phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), mitogen-activated/extracellular signal-regulated kinase kinase (MEK)1/2, phospho-MEK1/2 (Ser<sup>217/221</sup>), p38 MAPK, phospho-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>), AMP-activated protein kinase (AMPK), phospho-AMPK (Thr<sup>172</sup>) (Cell Signaling Technology, Beverly, MA), IRS-1, the p85 regulatory subunit of PI 3-kinase, p21Ras (Upstate Biotechnology, Lake Placid, NY), Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-IRS-1 (Ser<sup>312</sup>) (gift of Dr. M. White, Joslin Diabetes Center, Boston, MA), GLUT1 (Chemicon, Temecula, CA), and appropriate secondary antibodies (goat anti-rabbit and -mouse horseradish peroxidase conjugates; Bio-Rad Laboratories). In immunoprecipitation experiments, lysates were incubated with antibodies to Shc (Upstate Biotechnology) or IRS-1 or -2 (gift of Dr. M. White) at 4°C and coupled to protein A sepharose beads (Amersham Pharmacia Biotech, Piscata-

TABLE 1  
Characteristics of subjects

	Control	PCOS
<i>n</i>	15	20
Age (years)	33 ± 1	31 ± 1
BMI (kg/m <sup>2</sup> )	35.4 ± 1.4	38.1 ± 1.7
Total testosterone (nmol/l)	0.8 ± 0.1	2.7 ± 0.2*
Unbound testosterone (nmol/l)	0.17 ± 0.02	0.83 ± 0.04*
DHEAS ( $\mu$ mol/l)	4.1 ± 0.3	5.5 ± 0.6
Glucose (mmol/l)		
Fasting	4.7 ± 0.1	4.9 ± 0.1
2 h post-75 g glucose	5.4 ± 0.2	7.8 ± 0.4*
Insulin (pmol/l)		
Fasting	121 ± 14	196 ± 29†
2 h post-75 g glucose	395 ± 69	1,278 ± 206*

Data are means ± SE. \* $P < 0.0001$ , † $P < 0.05$ , PCOS vs. control subjects. DHEAS, dehydroepiandrosterone sulfate.

way, NJ) before SDS-PAGE and immunoblotting with antibodies to insulin receptor- $\beta$  (Transduction Laboratories, San Diego, CA) or Grb2 (Upstate Biotechnology). Immunoblots were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantitated using a scanning densitometer (Bio-Rad Laboratories). The same internal standard was loaded on all immunoblots and results expressed as percentage of internal standard. **ERK1/2 kinase assay.** ERK1/2 activity was measured using a kit (Cell Signaling Technology). Briefly, ERK1/2 was immunoprecipitated from lysates (200  $\mu$ g) and then incubated in buffer containing ATP and Ets-like protein 1-fusion protein (30 min, 30°C). The reaction was terminated by boiling in reducing sample buffer. The product (phospho-ETS-like protein 1) was resolved by SDS-PAGE and detected by immunoblotting.

**p21Ras-binding assay.** To determine p21Ras activity, p21Ras-binding to Raf-1 was measured using a kit (Upstate Biotechnology). Briefly, myotubes were lysed in 25 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 10% glycerol, 25 mmol/l NaF, 10 mmol/l MgCl<sub>2</sub>, 1 mmol/l EDTA, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml leupeptin, and 10  $\mu$ g/ml aprotinin and incubated with Raf-1 Ras-binding domain agarose for 30 min at 4°C. Ras bound to Raf-1 Ras-binding domain was resolved by SDS-PAGE and immunoblotted with anti-p21Ras antibody.

**MEK1/2 inhibitor studies.** Myotubes were incubated for 18 h with the selective noncompetitive inhibitor of MEK1/2, U0126 (20  $\mu$ mol/l; Cell Signaling Technology) (21), in fusion medium and then incubated for a further 2 h in serum-free medium. Replicate plates were incubated with vehicle (DMSO) alone. Cell lysates were prepared after treatment with or without insulin (100 nmol/l, 10 min) as above.

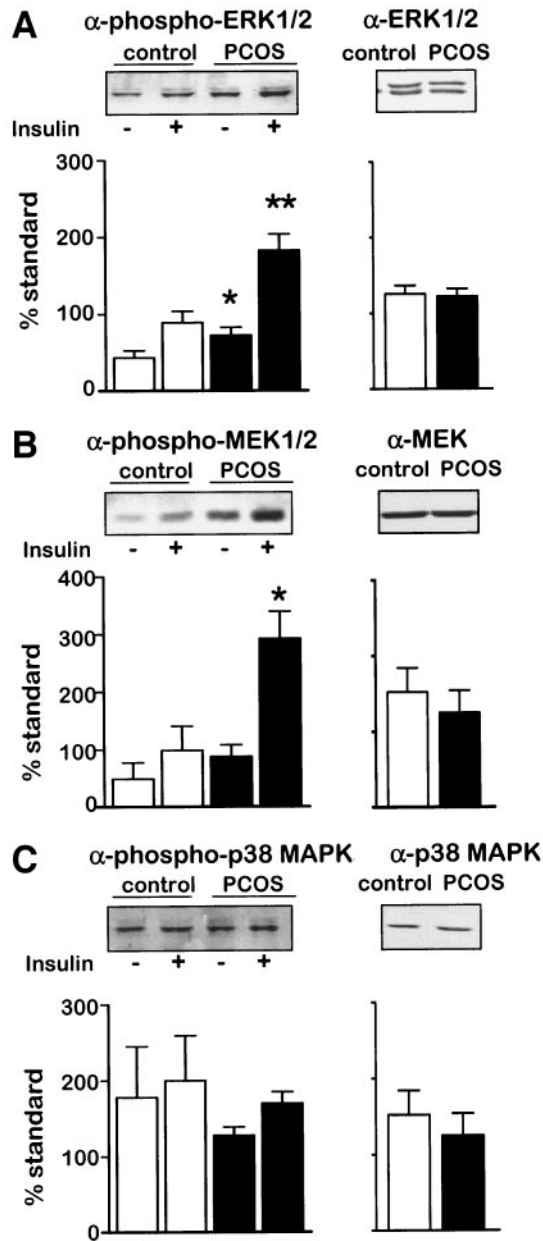
**Glucose transport assay.** Glucose uptake in three replicate wells for each condition was measured in myotubes incubated in serum-free medium for 2 h (22). Specific glucose transport was calculated in each well by measuring [<sup>3</sup>H]-deoxy-D-glucose (NEN Life Science, Boston, MA) incorporation and subtracting L-[<sup>14</sup>C]-glucose (NEN Life Science) incorporation to correct for non-GLUT-mediated glucose uptake.

**Statistical analysis.** PCOS and control subjects were compared using paired and unpaired *t* tests or nonparametric tests (Mann-Whitney for unpaired, Wilcoxon signed rank for paired) when the size of the sample group was six or fewer. Data are presented as mean of percent of internal standard ± SE, and differences were considered significant at  $P < 0.05$ .

## RESULTS

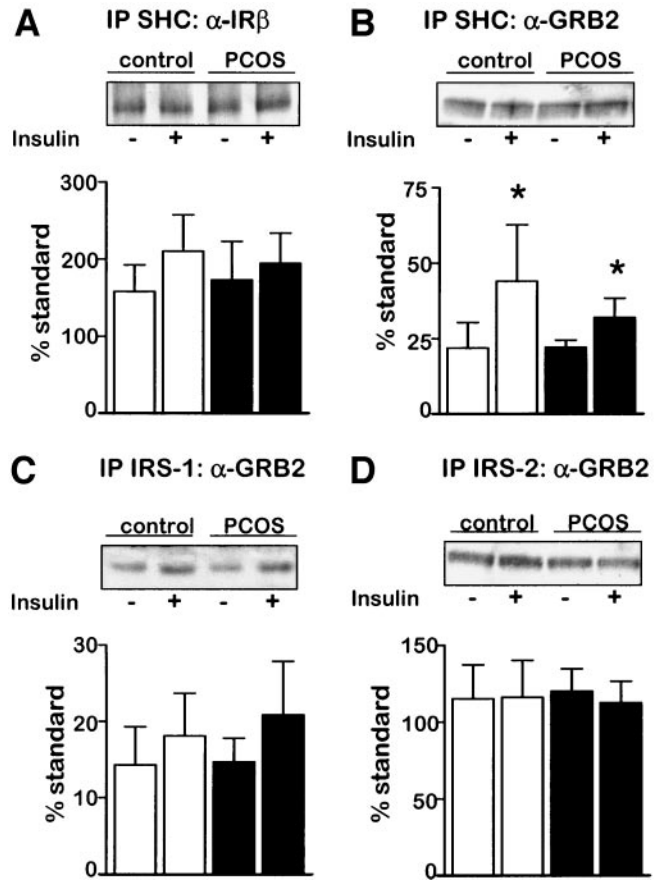
Women with PCOS had significant elevations of serum total testosterone and non-sex hormone-binding globulin-bound testosterone consistent with the biochemical profile of the disorder (1). Glucose and insulin 2 h post-75-g glucose load were significantly elevated in women with PCOS, consistent with the presence of insulin resistance. None of the subjects had type 2 diabetes. However, eight women with PCOS had impaired glucose tolerance (Table 1).

**Mitogenic signaling in cultured myotubes.** In myotubes from women with PCOS, baseline phospho-ERK1/2 was ~70% higher ( $P < 0.05$ ) and insulin-stimulated phospho-ERK1/2 ~100% higher than in control myotubes ( $P <$



**FIG. 1.** ERK1/2, MEK1/2, and p38 MAPK activation in PCOS (■) and control (□) myotubes with or without 100 nmol/l insulin for 10 min. Resolved lysates were immunoblotted with specific antibodies to phospho-ERK1/2 and ERK1/2 ( $n = 11$ /group) (A), phospho-MEK1/2 and MEK1/2 ( $n = 6$ /group) (B), and phospho-p38 MAPK and p38 MAPK ( $n = 6$ /group) (C). \* $P < 0.05$ , \*\* $P < 0.01$ , PCOS vs. control under the same conditions.

0.01) (Fig. 1A). Insulin increased phospho-ERK1/2 to a similar extent in PCOS ( $P < 0.001$  vs. baseline) and in control myotubes ( $P < 0.005$  vs. baseline). Consistent with other studies on human skeletal muscle (23), the predominant product was phospho-ERK2, although phospho-ERK1 was also detectable. The abundance of ERK1/2 protein did not differ in the two groups (Fig. 1A). Basal phospho-MEK1/2 tended to be higher in myotubes from women with PCOS than control subjects (Fig. 1B). Insulin-stimulated phospho-MEK1/2 was ~200% higher in PCOS than control subjects ( $P < 0.05$ ) (Fig. 1B). MEK1/2 protein abundance did not differ (Fig. 1B). To confirm that the increase in phospho-ERK1/2 in PCOS reflected increased activity of the kinase, ERK1/2 activity was assayed in the

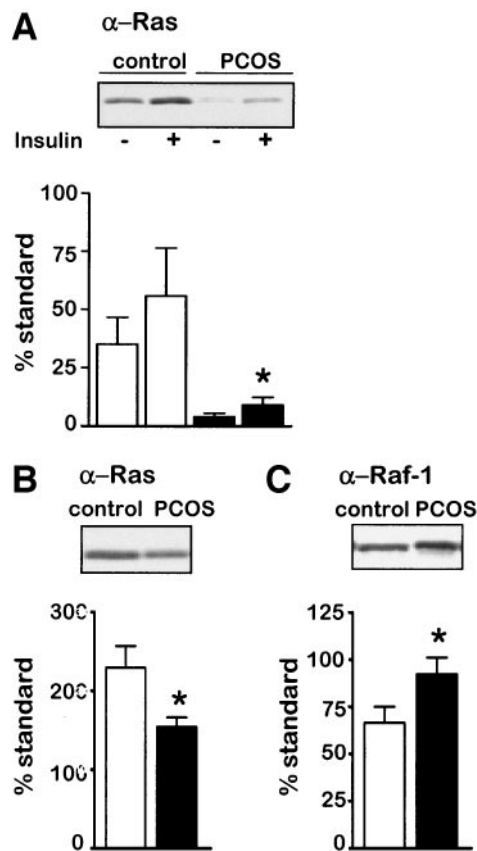


**FIG. 2.** Insulin mitogenic signaling upstream of p21Ras in PCOS (■) and control (□) myotubes with or without 100 nmol/l insulin for 10 min. Lysates immunoprecipitated with α-Shc were immunoblotted with α-insulin receptor (IR)-β ( $n = 6$ /group) (A) or α-Grb2 ( $n = 6$ /group) (B). Lysates immunoprecipitated with α-IRS-1 ( $n = 6$ /group) (C) or α-IRS-2 ( $n = 6$ /group) (D) were immunoblotted with α-Grb2. \* $P < 0.05$ , insulin stimulated vs. baseline.

same myotube lysates ( $n = 3$  PCOS,  $n = 4$  control), and results were comparable to phospho-ERK1/2 levels on the corresponding immunoblots (data not shown).

To determine if increased ERK1/2 phosphorylation in the myotubes of women with PCOS represented general activation of MAPK pathways, we assessed p38 MAPK phosphorylation in the same lysates (Fig. 1C). The abundance of phospho- and total p38 MAPK did not differ in the two groups at baseline. Following insulin stimulation, phospho-p38 MAPK abundance did not increase significantly in either group.

**ERK1/2 signaling upstream of p21Ras.** We examined protein interactions in the proximal insulin signaling pathway to investigate the mechanism for increased activation of ERK1/2 in myotubes in PCOS. The abundance of insulin receptor-β in Shc immunoprecipitates did not differ in the two groups at baseline or following insulin stimulation (Fig. 2A). Similarly, the abundance of Grb2 in Shc immunoprecipitates did not differ (Fig. 2B). Insulin stimulated the association of Shc with Grb2 to a similar extent ( $P < 0.05$ , insulin treated vs. baseline) in both groups (Fig. 2B). The abundance of Grb2 in both IRS-1 (Fig. 2C) and IRS-2 (Fig. 2D) immunoprecipitates did not differ in the two groups at baseline or following insulin stimulation. Thus, no abnormalities were found in the signaling pathway from the insulin receptor to Grb2/Sos, mediated by IRS-1, IRS-2, or Shc in myotubes from women with PCOS.



**FIG. 3.** Insulin mitogenic signaling via p21Ras and Raf-1. Ras activity assessed in PCOS (■) and control (□) myotubes with or without 100 nmol/l insulin for 10 min by incubating lysates with Raf-1 Ras-binding domain conjugated to agarose beads ( $n = 4/\text{group}$ ). Resolved products were immunoblotted with  $\alpha$ -p21Ras (A). The abundance of p21Ras (B) and Raf-1 (C) was assessed by immunoblotting with specific antibodies ( $n = 11/\text{group}$ ). \* $P < 0.05$ , PCOS vs. control under the same conditions.

**Signaling via p21Ras and Raf-1.** Activity of p21Ras, as assessed by binding to Raf-1, tended to be lower at baseline and was  $\sim 80\%$  lower following insulin stimulation ( $P < 0.05$ ) in myotubes from women with PCOS than in control subjects (Fig. 3A). The abundance of p21Ras was  $\sim 30\%$  lower in PCOS ( $P < 0.05$ ) (Fig. 3B). These results suggested that decreased p21Ras abundance is a factor contributing to the reduced p21Ras activity in myotubes from women with PCOS. Thus, increased activation of MEK/ERK1/2 in myotubes from women with PCOS could not be explained by changes in p21Ras activity. However, Raf-1 abundance was significantly increased ( $P < 0.05$ ) (Fig. 3C) and the electrophoretic mobility of Raf-1 retarded in myotubes from women with PCOS (Fig. 3C), consistent with the altered phosphorylation state of this protein.

**Mitogenic signaling in skeletal muscle tissue.** We examined signaling from p21Ras to ERK1/2 in muscle biopsies to determine if the abnormalities in myotubes of women with PCOS reflected changes in skeletal muscle in vivo. The abundance of phospho-ERK1/2 was  $\sim 100\%$  higher at baseline ( $P < 0.05$ ) in skeletal muscle tissue from women with PCOS, and total ERK1/2 abundance did not differ (Fig. 4A). Basal phospho-MEK1/2 tended to be higher in PCOS (Fig. 4B). MEK protein abundance did not differ in the two groups (Fig. 4B). The abundance of phospho-p38 MAPK did not differ in the two groups (Fig. 4C). The abundance of p38 MAPK protein was also similar (Fig. 4C).

The abundance of p21Ras did not differ in the two groups (Fig. 4D); however, Ras-binding activity was not assessed due to limited tissue availability. The abundance of Raf-1 protein did not differ significantly (Fig. 4E). Thus, with the exception of p21Ras abundance, the abnormalities in the skeletal muscle tissue from women with PCOS were similar to those observed in cultured myotubes.

**Effect of MEK inhibition.** We previously reported that cultured myotubes of women with PCOS displayed constitutively increased phosphorylation of IRS-1 on Ser<sup>312</sup> (9). Phosphorylation at this key regulatory site leads to attenuation of insulin signaling (24–26). We investigated the effect of MEK inhibition to determine whether enhanced ERK1/2 signaling was causally related to increased IRS-1 Ser<sup>312</sup> phosphorylation in PCOS. As expected, incubation of cultured myotubes from PCOS or control women with U0126 resulted in complete inhibition of ERK1/2 phosphorylation (Fig. 5A) with no change in ERK1/2 protein abundance (Fig. 5B). Consistent with our previous studies (9), IRS-1 protein abundance tended to be higher and phosphorylation of IRS-1 on Ser<sup>312</sup> was  $\sim 80\%$  higher ( $P = 0.06$ ) at baseline in the myotubes of women with PCOS, but these changes did not reach statistical significance in the current small group of subjects (Fig. 5C–E). Adjusting phospho-IRS-1 Ser<sup>312</sup> for total IRS-1 abundance did not affect the results. U0126 treatment did not alter IRS-1 protein abundance (Fig. 5D), but basal phosphorylation of IRS-1 Ser<sup>312</sup> was reduced by a similar amount (50–60%) in both groups ( $P < 0.05$ , U0126 treated vs. no U0126) (Fig. 5C and E). U0126 treatment reduced basal phosphorylation of IRS-1 Ser<sup>312</sup> in PCOS to values similar to those in control myotubes. Insulin-stimulated phosphorylation of IRS-1 Ser<sup>312</sup> was similarly reduced by U0126 treatment in both groups ( $P < 0.05$ , U0126 treated vs. no U0126) (Fig. 5C and E). The association of IRS-1 with p85 increased to a similar extent (100–150%) after U0126 treatment alone ( $P < 0.05$ , U0126 treated vs. no U0126) and in combination with insulin ( $\sim 100\%$ ) ( $P < 0.05$ , U0126 treated vs. no U0126) in myotubes from both PCOS and control women (Fig. 5G and H). However, the magnitude of the response to insulin was unchanged from that seen in the absence of U0126 in both groups. U0126 treatment did not alter the abundance of p85 protein (Fig. 5F).

Treatment with U0126 increased basal and insulin-stimulated glucose transport ( $P < 0.05$ ) (Fig. 6A). The effect of U0126 on basal glucose transport was not mediated by a change in GLUT1 protein abundance (Fig. 6B and C). The abundance of GLUT4 protein was also unchanged by U0126 treatment (data not shown). However, U0126 alone increased phosphorylation of the catalytic subunit of AMPK to a similar extent ( $\sim 80\%$ ) in both groups, but this change did not achieve statistical significance (Fig. 6D and F). Phospho-AMPK abundance at baseline tended to be higher in the myotubes of women with PCOS ( $P = 0.06$ ) (Fig. 6F), whereas insulin did not alter its abundance in the presence or absence of U0126 in either group (Fig. 6D and F). U0126 treatment did not affect total AMPK protein abundance (Fig. 6E).

## DISCUSSION

The present study has shown increased activation of the ERK1/2 pathway in skeletal muscle in PCOS. This abnormality was present in freshly isolated muscle tissue, suggesting that it is not an artifact of cell culture. Proximal signaling via IRS-1/2 or Shc to p21Ras was similar in both

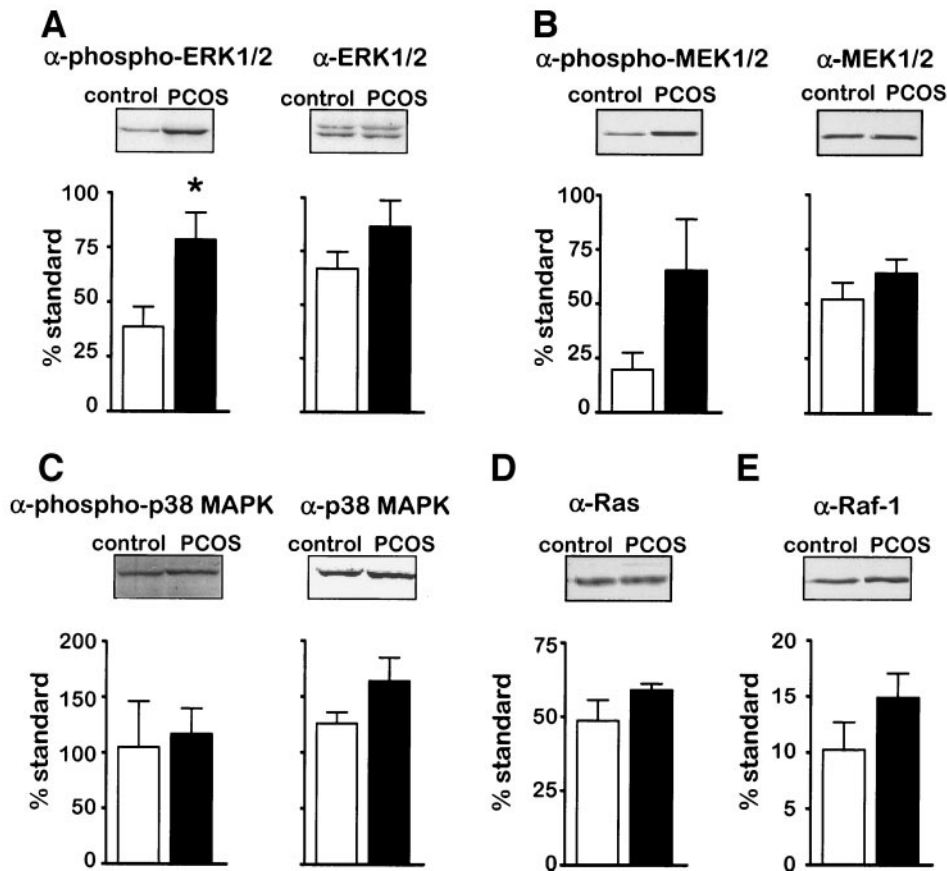


FIG. 4. Mitogenic signaling in skeletal muscle tissue from PCOS (■) and control (□) women. Resolved lysates were immunoblotted with specific antibodies to phospho-ERK1/2 and ERK1/2 ( $n = 7/\text{group}$ ) (A), phospho-MEK1/2 and MEK1/2 ( $n = 7$  control subjects,  $n = 10$  PCOS) (B), phospho-p38 MAPK and p38 MAPK ( $n = 6$  control subjects,  $n = 7$  PCOS) (C), Ras ( $n = 5$  control subjects,  $n = 6$  PCOS) (D), and Raf-1 ( $n = 5/\text{group}$ ) (E). \*  $P < 0.05$ , PCOS vs control.

groups of myotubes. However, p21Ras activity was decreased, in part due to decreased protein abundance, and Raf-1 abundance was increased in myotubes from women with PCOS. Since MEK activity was increased and p21Ras activity was decreased, it is likely that activation of this pathway began at the level of Raf-1 in PCOS. The enhanced ERK1/2 phosphorylation in PCOS did not reflect generalized activation of MAPK pathways, as p38 MAPK phosphorylation in skeletal muscle in vitro and in vivo did not differ between women with PCOS and control women. In contrast to our findings in PCOS, skeletal muscle tissue in type 2 diabetes is characterized by increased p38 MAPK phosphorylation (27), and, in isolated adipocytes from patients with type 2 diabetes, there is a generalized increase in MAPK phosphorylation, especially p38 MAPK (28). The present findings are thus consistent with our previous studies, which suggested that PCOS represents a unique subphenotype of insulin resistance (4,9).

There is considerable evidence that the MEK/ERK1/2 signaling pathway is involved in serine phosphorylation of IRS-1 (25,29–32), resulting in decreased IRS-1 tyrosine phosphorylation and inhibition of association with PI 3-kinase (33), a critical step for propagating the insulin signal to metabolic pathways (10,11). Our studies with the MEK inhibitor U0126 in human myotubes are consistent with these findings. Moreover, our data suggest that this pathway participates in the feedback regulation of metabolic signaling under normal circumstances, as well as in PCOS, since phosphorylation at IRS-1 Ser<sup>312</sup> was decreased and association of the p85 regulatory subunit of PI 3-kinase was increased during MEK inhibitor treatment in both groups of myotubes. Phosphorylation of IRS-1 Ser<sup>312</sup> impairs signaling by decreasing docking of the p85 regu-

latory subunit of PI 3-kinase (24–26), inhibiting interaction with the insulin receptor (26) and promoting degradation of IRS-1 (34). MEK inhibition reduced phosphorylation to control levels, suggesting that ERK1/2 or ERK1/2-regulated kinases are responsible for constitutive phosphorylation of IRS-1 Ser<sup>312</sup> in PCOS myotubes (9). Whether ERK1/2 directly associates with IRS-1 (29) or activates other serine kinases that phosphorylate IRS-1 Ser<sup>312</sup> is unknown (24,32,35,36). Although prolonged incubation (18 h) with U0126 in the current study did not change the abundance of any of the key signaling proteins measured, it is possible that altered expression of other proteins could have contributed to the increase in signaling via IRS-1. Since insulin-stimulated glucose uptake is decreased in vivo but not in cultured myotubes from women with PCOS, additional circulating factors are involved in the pathogenesis of insulin resistance in this syndrome (9).

Nevertheless, these observations suggest that enhanced signaling through mitogenic pathways could contribute to impaired insulin metabolic signaling in PCOS. Constitutive activation of ERK1/2 in skeletal muscle, exacerbated by hyperinsulinemia resulting from resistance to insulin's metabolic actions, would result in increased phosphorylation of IRS-1 Ser<sup>312</sup>, thus decreasing insulin-mediated glucose uptake in vivo in PCOS (37). Selective insulin resistance with intact insulin action on mitogenic pathways has been reported in cultured skin fibroblasts in PCOS (13) and type 2 diabetes (14) and in skeletal muscle tissue in type 2 diabetes (15,16), as well as in the vasculature of Zucker fatty rats (38). Although constitutive activation of ERK1/2 has not been reported in skeletal muscle in vivo in type 2 diabetes (15,16), such activation has recently been reported in adipocytes (28) and in

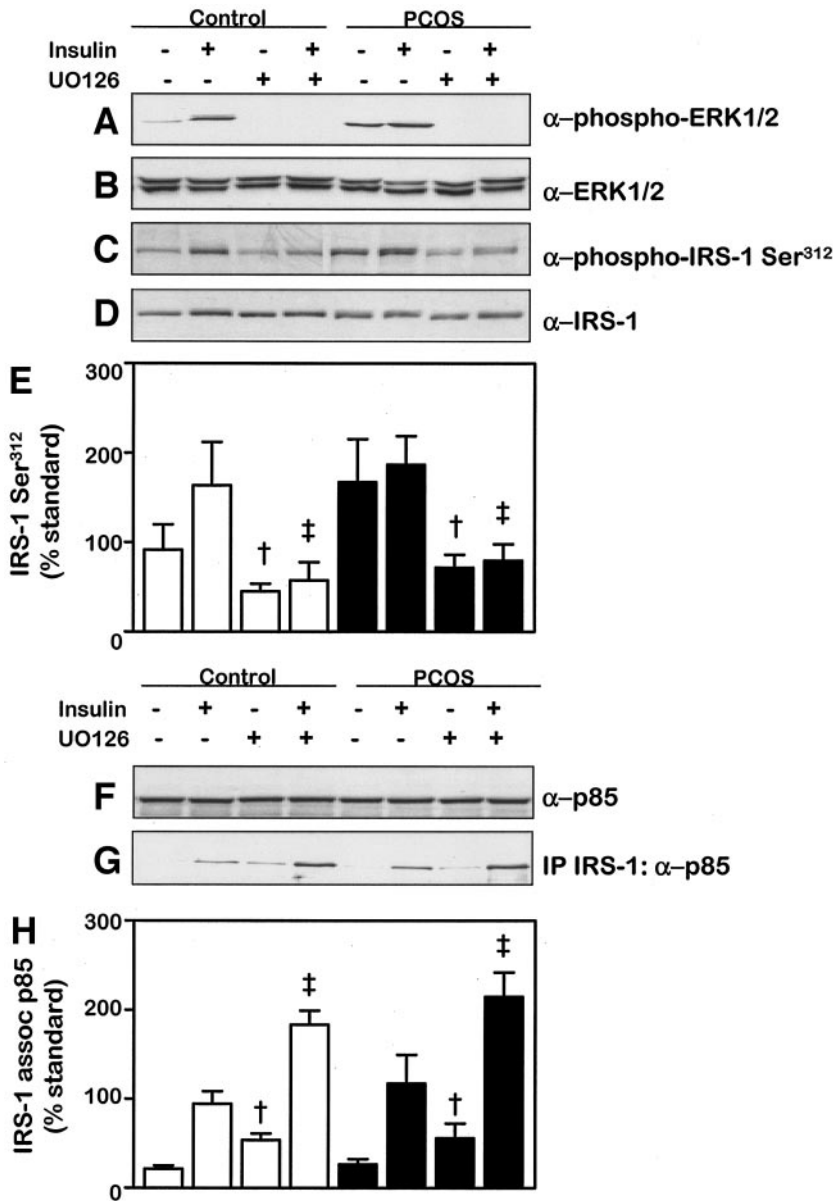


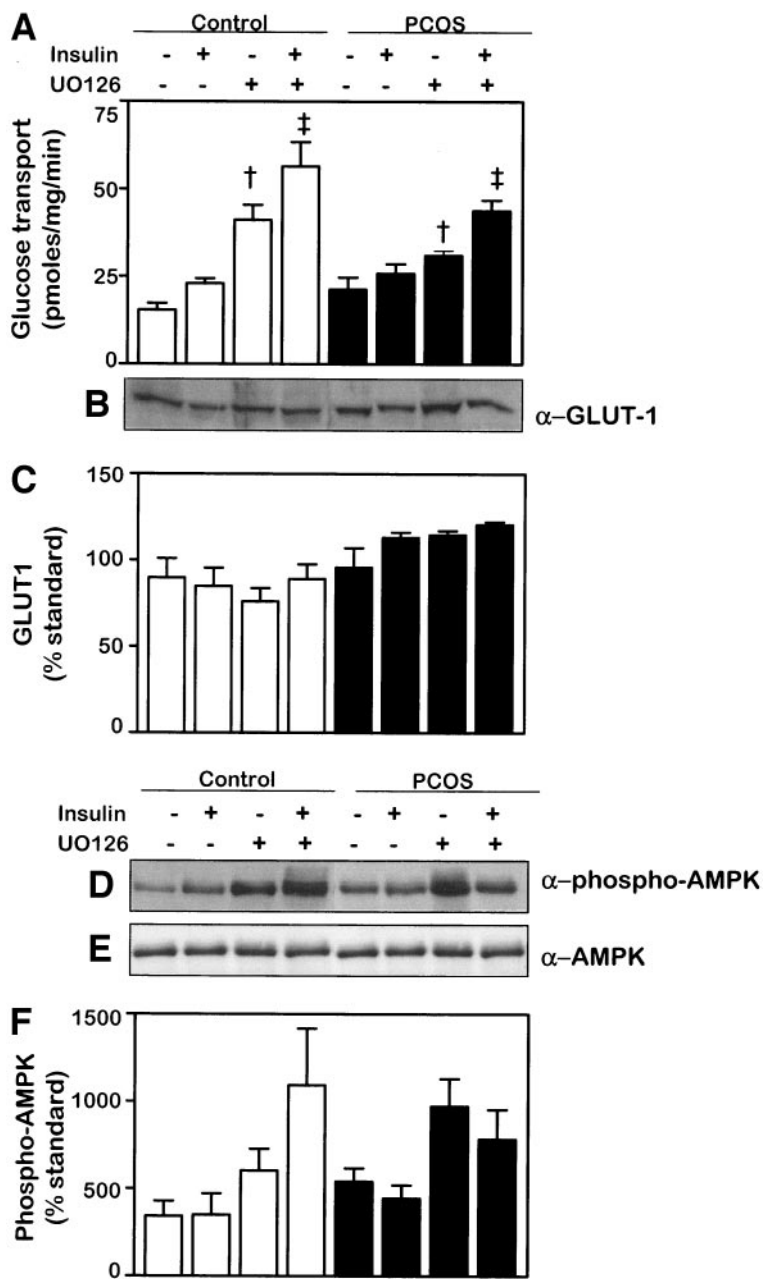
FIG. 5. Effect of MEK inhibition on insulin mitogenic and metabolic signaling in PCOS and control myotubes. Myotubes were preincubated with U0126 for 18 h and then treated with or without 100 nmol/l insulin for 10 min. Resolved lysates were immunoblotted with specific antibodies to phospho-ERK1/2 (*n* = 6 control subjects, *n* = 7 PCOS) (*A*), ERK1/2 (*B*), phospho-IRS-1 Ser<sup>312</sup> (*n* = 6 control subjects [□], *n* = 7 PCOS [■]) (*C* and *E*), and IRS-1 (*D*). The abundance of p85 in total lysates (*F*) and in IRS-1 immunoprecipitates (*G* and *H*) was also assessed by immunoblotting (*n* = 6/group). †*P* < 0.05, basal U0126 treated vs. basal non-U0126 treated; ‡*P* < 0.05, insulin-stimulated U0126 treated vs. insulin-stimulated non-U0126 treated.

cultured myotubes (39) from affected subjects. Furthermore, the latter study demonstrated that ERK1/2 activation contributed to increased phosphorylation of IRS-1 on Ser<sup>636</sup>, another inhibitory site for signaling (40). It is thus possible that selective insulin resistance with preserved mitogenic signaling resulting in phosphorylation of IRS-1 on serine sites that impair metabolic signaling may be a more general mechanism contributing to human insulin resistance.

In cultured myotubes from women with PCOS, no abnormalities were found in signaling from the insulin receptor to p21Ras. However, increased Raf-1 protein abundance as well as a mobility shift consistent with an altered phosphorylation state of this protein was found, suggesting that the increased mitogenic signaling in PCOS was initiated at this level of the pathway. We cannot exclude the possibility that altered Raf-1 phosphorylation in PCOS was inhibitory to signaling (41). Another explanation for the absence of abnormalities in upstream signaling to the ERK1/2 pathway is the existence of alternative pathways involving protein kinase C that by-

pass IRS, Shc, and p21Ras (42,43). Our findings are in contrast to those reported in peripheral blood mononuclear cells in women with PCOS, where p21Ras protein abundance and activity, as well as basal and insulin-stimulated ERK1/2 phosphorylation, were unchanged (44), illustrating tissue specificity of defects in PCOS (9,13).

The MEK inhibitor studies also permitted us to determine if ERK1/2 played a role in the increased non-insulin-mediated glucose transport and GLUT1 protein abundance that we previously reported in myotubes of women with PCOS (9), analogous to the effects of increased ERK1/2 expression in 3T3-L1 adipocytes (45). Surprisingly, U0126 significantly increased non-insulin-mediated glucose transport in myotubes both from women with PCOS and from control subjects, suggesting that ERK1/2 was inhibitory to glucose transport. This effect of U0126 was not mediated by a change in abundance of GLUT1 or -4 protein. To resolve the apparent paradox, we investigated U0126 effects on AMPK, an important regulator of insulin-independent glucose uptake (46). We found that U0126-stimulated AMPK phosphorylation in the myotubes of



**FIG. 6.** Effect of MEK inhibition on glucose transport and AMPK phosphorylation in PCOS (■) and control (□) myotubes. Glucose transport ( $n = 4$ /group) (A) and GLUT1 abundance ( $n = 3$ /group) (B and C) were measured in myotubes preincubated with UO126 for 18 h. Phospho- and total AMPK abundance were measured in myotubes preincubated with or without UO126 for 18 h and then treated with or without 100 nmol/l insulin for 10 min. Resolved lysates ( $n = 5$ /group) were immunoblotted with specific antibodies to phospho-AMPK (D and F) and AMPK (E). † $P < 0.05$ , basal UO126 treated vs. basal non-UO126 treated; ‡ $P < 0.05$ , insulin-stimulated UO126 treated vs. insulin-stimulated non-UO126 treated.

women with PCOS and control subjects, and thus this mechanism rather than MEK inhibition could account for the increase in basal glucose transport observed with UO126. Our results are consistent with a recent report that UO126 stimulates AMPK activity independent of MEK inhibition (47).

Enhanced activation of mitogenic/gene-regulatory pathways could contribute to other features of the PCOS phenotype. Activation of ERK1/2 might account for the increase in markers of myogenic differentiation that we previously reported in myotubes from women with PCOS (9), since this pathway plays an important role in the initiation and progression of human myoblast differentiation (48). It has been suggested that selective insulin resistance also contributes to the paradoxical effects of insulin on steroidogenesis in PCOS (1). Little is known about the insulin signaling pathways modulating ovarian androgen biosynthesis; however, significantly decreased ERK1/2 activation has recently been reported in PCOS

theca cells (49). Although the activation of the mitogenic signaling pathways in PCOS skeletal muscle represents a stable phenotype that persists in long-term cell culture, it is unknown whether this change is a genetic trait or represents programming by environmental factors, such as androgens (9,50).

In conclusion, the ERK1/2 signaling pathway is constitutively increased in the skeletal muscle of women with PCOS both in vivo and in cultured cells. Kinases extrinsic to the insulin receptor have been implicated in the pathogenesis of the postbinding defect in signaling in PCOS and the present study suggests that ERK1/2 or ERK1/2-regulated kinases are responsible for the increased phosphorylation of IRS-1 Ser<sup>312</sup>. ERK1/2 also modulates the phosphorylation of IRS-1 Ser<sup>312</sup> in myotubes from control women, suggesting that this pathway participates in the feedback regulation of insulin signaling under normal circumstances. However, abnormal activation of this pathway in the skeletal muscle of women with PCOS may

contribute to the defects in insulin metabolic signaling in this disorder.

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