TRANSLATIONAL PHYSIOLOGY

Insulin resistance in the skeletal muscle of women with PCOS involves intrinsic and acquired defects in insulin signaling

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Corbould, Anne, Young-Bum Kim, Jack F. Youngren, Celia Pender, Barbara B. Kahn, Anna Lee, and Andrea Dunaif. Insulin resistance in the skeletal muscle of women with PCOS involves intrinsic and acquired defects in insulin signaling. Am J Physiol Endocrinol Metab 288: E1047–E1054, 2005. First published December 21, 2004; doi:10.1152/ajpendo.00361.2004.—Insulin resistance in polycystic ovary syndrome (PCOS) is due to a postbinding defect in signaling that persists in cultured skin fibroblasts and is associated with constitutive serine phosphorylation of the insulin receptor (IR). Cultured skeletal muscle from obese women with PCOS and age- and body mass index-matched control women (n = 10/group) was studied to determine whether signaling defects observed in this tissue in vivo were intrinsic or acquired. Basal and insulin-stimulated glucose transport and GLUT1 abundance were significantly increased in cultured myotubes from women with PCOS. Neither IR β-subunit abundance and tyrosine autophosphorylation nor insulin receptor substrate (IRS)-1-associated phosphatidylinositol (PI) 3-kinase activity differed in the two groups. However, IRS-1 protein abundance was significantly increased in PCOS, resulting in significantly decreased PI 3-kinase activity when normalized for IRS-1. Phosphorylation of IRS-1 on Ser312, a key regulatory site, was significantly increased in PCOS, which may have contributed to this signaling defect. Insulin signaling via IRS-2 was also decreased in myotubes from women with PCOS. In summary, decreased insulin-stimulated glucose uptake in PCOS skeletal muscle in vivo is an acquired defect. Nevertheless, there are intrinsic abnormalities in glucose transport and insulin signaling in myotubes from affected women, including increased phosphorylation of IRS-1 Ser312, that may confer increased susceptibility to insulin resistance-inducing factors in the in vivo environment. These abnormalities differ from those reported in other insulin resistant states consistent with the hypothesis that PCOS is a genetically unique disorder conferring an increased risk for type 2 diabetes.

Polycystic ovary syndrome; myotubes; glucose transport; glucose transporter 1; insulin receptor substrate; serine phosphorylation

Polycystic ovary syndrome (PCOS) is a common disorder of premenopausal women characterized by disordered gonadotropin secretion, hyperandrogenism, and a substantially increased risk for type 2 diabetes mellitus (T2DM) (16, 37). Women with PCOS have profound peripheral insulin resistance, independent of obesity, due to a postbinding defect in insulin signaling (9, 11–13). The molecular mechanisms of this defect are distinctive. In insulin receptors (IR) isolated from cultured skin fibroblasts of ~50% of women with PCOS, there is constitutive serine phosphorylation that inhibits insulin-stimulated tyrosine phosphorylation (14). Recent studies have confirmed that a serine kinase extrinsic to the IR inhibits its autophosphorylation in PCOS (14, 38). The resulting insulin resistance is selective, affecting metabolic but not mitogenic pathways (5). The mechanism for insulin resistance in the PCOS skin fibroblasts with normal IR serine phosphorylation has not been investigated but may involve serine phosphorylation of downstream signaling molecules such as insulin receptor substrate (IRS)-1. In skeletal muscle, the major site of insulin-mediated glucose uptake, IRS-1-associated phosphatidylinositol (PI) 3-kinase activity is decreased in women with PCOS in vivo, consistent with a defect in IR- or IRS-1-mediated signaling (13). Insulin resistance clusters in PCOS families (36), and defects in insulin action persist in cultured skin fibroblasts of women with PCOS (5, 14), suggesting that there is genetic susceptibility to these abnormalities.

In this study, we examined insulin action on glucose metabolism and IR signaling in cultured myotubes from women with PCOS to determine whether the defects that we detected in acutely isolated skeletal muscle were intrinsic. Cultured human skeletal muscle is a well-validated system that maintains insulin responsiveness and glucose transporter 4 (GLUT4) expression (6, 8, 21, 25, 26, 28, 34, 46, 48–51). Parameters of in vivo insulin action have been significantly correlated with insulin action in cultured skeletal muscle cells from Pima Indians (48, 51). This culture system has been used to investigate the presence of intrinsic defects in insulin action in individuals with T2DM (6, 8, 21, 25, 26) or impaired glucose tolerance (IGT) (49) as well as in first-degree relatives of patients with T2DM (28, 34).

We hypothesized that the cultured skeletal muscle of women with PCOS would be insulin resistant, in keeping with a...
genetically determined defect in insulin signaling. We found that, despite defects in insulin signaling via IRS-1 and IRS-2, the cultured myotubes from women with PCOS showed normal insulin responsiveness, consistent with a major role of the metabolic/hormonal environment in the pathogenesis of in vivo insulin resistance in this syndrome. Moreover, many of the phenotypic features of these myotubes differed from those reported in other insulin resistant states consistent with the hypothesis that PCOS is a genetically unique subphenotype of insulin resistance.

MATERIALS AND METHODS

Subjects. The study was approved by the Institutional Review Boards of Brigham and Women’s Hospital and of Northwestern University; all subjects gave written, informed consent. Ten obese women with PCOS and 10 age-, body mass index (BMI), and ethnicity-matched control women were studied. Women were aged 22–42 years, in good health, and taking no medications known to affect carbohydrate or sex hormone metabolism for ≥1 mo before the study except for oral contraceptive agents, which were discontinued 3 mo before study. Control women had menses every 27–35 days, no clinical or biochemical evidence of hyperandrogenism, normal glucose tolerance, and no first-degree relatives with T1DM or T2DM.

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control) and treatment status (with vs. without insulin) as factors. Simple linear regressions were used to calculate Pearson’s correlation coefficient r. Data were log transformed when necessary to achieve homogeneity of variance. Data are reported as untransformed means ± SE with significance at P < 0.05.

RESULTS

Subject characteristics. By design, the two groups of subjects were matched for age and BMI (Table 1). There were significant elevations in total and biologically available testosterone levels as well as in glucose and insulin levels at baseline and 2 h post-75-g glucose load, consistent with the presence of insulin resistance, in women with PCOS. None of the subjects had T2DM. However, six women with PCOS fulfilled WHO criteria for IGT.

Assessment of myotube growth and differentiation. Population doubling times did not differ [1.9 ± 0.2 PCOS vs. 1.9 ± 0.2 control, days/doubling, n = 5/group, P = not significant (NS)] in the two groups of myoblasts. After 4 days in fusion medium, consistently greater than 85% of nuclei were within multinucleated cells (i.e., myotubes) from both groups of muscle cells. However, CK activity was significantly higher in myotubes from women with PCOS (3,077 ± 520 PCOS vs. 1,030 ± 125 control, Sigma units·ml⁻¹·ng protein⁻¹, n = 10/group, P < 0.01). Additionally, the abundance of MHC was significantly increased in myotubes from women with PCOS (103 ± 14 PCOS vs. 34 ± 9 control, n = 6/group, P < 0.05). These findings suggested a greater degree of differentiation in myotubes from women with PCOS.

Glucose transport and glycogen synthesis. Glucose transport was ~60% higher in myotubes from women with PCOS at baseline (P < 0.05) and following maximal insulin stimulation (P < 0.05; Fig. 1A). However, the increment in glucose transport did not differ in the two groups. The fold stimulation of glucose transport (~1.5-fold) was similar to that of previous reports utilizing this cell culture system (8, 21, 25, 34, 46, 49).

Extending the incubation of control myotubes in fusion medium to 6 or 8 days compared with the standard 4 days did not change basal and insulin-stimulated glucose transport (Fig. 1C), despite significant increases in markers of differentiation (MHC abundance; Fig. 1D) and also CK activity (data not shown). Basal and insulin-stimulated glycogen synthesis was also ~60% higher in myotubes from women with PCOS, but this change did not reach statistical significance (P = 0.06; Fig. 1B). The increment and fold stimulation of glycogen synthesis did not differ in the two groups. The abundance of GLUT1 was ~50% higher in myotubes from women with PCOS (P < 0.05), whereas that of GLUT4 did not differ (Fig. 2A). As expected, GLUT1 abundance and basal glucose uptake were positively correlated (r = +0.6, P < 0.05; Fig. 2B).

Insulin signaling. The abundance of the following signaling proteins did not differ in the two groups: IRβ, IRS-2, and p85 regulatory subunit of PI 3-kinase (Fig. 3, A, C, and D). The electrophoretic mobility of IRS-2 was shifted in four of six

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Table 1. Characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>PCOS (n = 10)</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>34 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>36.2 ± 1.8</td>
<td>38.0 ± 1.5</td>
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<tr>
<td>Testosterone, nmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.8 ± 0.1</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Unbound</td>
<td>0.17 ± 0.03</td>
<td>0.87 ± 0.07*</td>
</tr>
<tr>
<td>DHEA-S, μmol/l</td>
<td>4.1 ± 0.4</td>
<td>5.4 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. PCOS, polycystic ovary syndrome; DHEAS, dehydroepiandrosterone sulfate. *P < 0.001, †P < 0.05, ‡P < 0.01, PCOS vs. control subjects.

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Fig. 1. Insulin action in cultured myotubes from age- and body mass index (BMI)-matched obese women with polycystic ovary syndrome (PCOS) and control women. A: glucose uptake (n = 8/group) was measured in myotubes at baseline and after incubation with insulin (1–100 nmol/l). B: glucose incorporation into glycogen (n = 10/group) was measured in myotubes at baseline and after incubation with insulin (1–100 nmol/l). C: effect of extending the duration of incubation in fusion medium on glucose uptake was assessed in the cultured muscle cells of a control subject. Open bars, basal glucose uptake; hatched bars, glucose uptake after stimulation with insulin (100 nmol/l). D: abundance of myosin heavy chain (MHC) was assessed using immunoblotting of confluent myoblasts before incubation in fusion medium (day 0) and in myoblasts incubated in fusion medium for 4, 6, or 8 days from the same control subject. Results are expressed relative to MHC abundance in cells incubated in fusion medium for 4 days (100%). Data are presented as means ± SE. *P < 0.05 by ANOVA, PCOS vs. control.
lysates from women with PCOS (Fig. 3C), suggesting post-translational modification. Basal autophosphorylation (Fig. 4A) and insulin-stimulated tyrosine phosphorylation of IRβ did not differ in the two groups (Fig. 4B). IRS-1 abundance was increased by 35% (P = 0.05), and its mobility was slightly reduced in myotubes from women with PCOS (Fig. 3B). Insulin-stimulated IRS-1 tyrosine phosphorylation, adjusted for total IRS-1 content, tended to be decreased in myotubes from women with PCOS, but this change did not reach significance (Fig. 4C). Similar amounts of IRS-1 were immunoprecipitated by the anti-IRS-1 antibody in both groups (data not shown). Without alterations in IRS-1 abundance being taken into account, IRS-1-associated PI 3-kinase activity and the association of p85 with IRS-1 (Fig. 5A) did not differ in the two groups. However, after adjustment for IRS-1 abundance, IRS-1-associated PI 3-kinase activity was decreased in myotubes from women with PCOS both at baseline (P = 0.05) and after insulin-stimulation (P < 0.01; Fig. 5B). Insulin-stimulated IRS-2-association with p85 was significantly decreased in myotubes from women with PCOS (P < 0.05; Fig. 5C). Baseline IRS-2-associated PI 3-kinase activity was significantly reduced (P < 0.05; Fig. 5D), and insulin-stimulated IRS-2-associated PI 3-kinase activity tended to be decreased (Fig. 5D) in myotubes from women with PCOS.

**Phosphorylation of IRS-1 Ser^312.** IRS-1 Ser^312 (Ser^307 in rat IRS-1) is a critical site, phosphorylation of which inhibits insulin-induced tyrosine phosphorylation of IRS-1 (2, 45). The abundance of IRS-1 phospho-Ser^312 was twofold higher (P < 0.01), consistent with the mobility shift in IRS-1 (Fig. 3C), in myotubes from women with PCOS at baseline. After adjustment for the ~35% increase in IRS-1 abundance, phosphorylation on IRS-1 Ser^312 was still ~55% higher in myotubes from women with PCOS (P < 0.01; Fig. 6).

**DISCUSSION**

In PCOS, decreased insulin-mediated glucose uptake in the major insulin target tissues, adipocytes and skeletal muscle, is due to a postbinding defect in insulin signaling when these tissues are examined shortly after isolation from the in vivo environment (9, 12, 13). However, in the present study, insulin-mediated glucose uptake at baseline and GLUT1 protein abundance in cultured myotubes (n = 8/group; r = +0.60; P < 0.05).

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**Fig. 2.** Abundance of glucose transporters GLUT1 (A) and GLUT4 (B) in total lysates (50 μg) of PCOS (P) and control (C) myotubes (n = 10/group) at baseline was determined by immunoblot analysis. Lysates were resolved by SDS-PAGE (10% gels), and representative immunoblots are shown. Filled bars, myotubes from women with PCOS; open bars, control myotubes. Data are presented as means ± SE. *P < 0.05 by paired t-test, PCOS vs control.

**Fig. 3.** Abundance of proximal insulin-signaling proteins in cultured myotubes from age- and BMI-matched obese women with PCOS and control women. Abundance of IRβ (A), IRS-1 (B), IRS-2 (C), and p85 (D) (n = 10/group for IRβ; IRS-1, and p85; n = 6/group for IRS-2) was determined by immunoblot analysis of PCOS (P) and control (C) myotubes. Basal lysates (50 μg) were separated by SDS-PAGE (7.5% gels), and representative immunoblots are shown. Filled bars, myotubes from women with PCOS; open bars, control myotubes. Data are presented as means ± SE. *P < 0.05 by paired t-test, PCOS vs control.
lin-mediated glucose uptake was not decreased in cultured myotubes from women with PCOS. This finding suggests that environmental factors play a major role in the pathogenesis of defects in insulin-mediated glucose uptake in this syndrome. Nevertheless, there were intrinsic changes in insulin signaling and glucose uptake in cultured myotubes from women with PCOS. Despite the fact that total IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity were comparable in the groups, IRS-1 protein abundance was increased in cultured myotubes from women with PCOS, so that IRS-1-associated PI 3-kinase activity adjusted for IRS-1 content was decreased. There was constitutively increased phosphorylation of IRS-1 Ser112 in myotubes from women with PCOS, even after adjustment for IRS-1 protein abundance. Phosphorylation at this key regulatory site (equivalent to Ser307 in rat IRS-1) inhibits insulin-induced tyrosine phosphorylation of IRS-1 and thus PI 3-kinase-dependent signaling pathways (2, 45). The upregulation in IRS-1 protein abundance normalized total IRS-1-associated PI 3-kinase activity suggested that this change was compensatory for an abnormality in IRS-1-mediated signaling. Basal IRS-2-associated PI 3-kinase activity and insulin-stimulated p85 association with IRS-2 were decreased in myotubes from women with PCOS, suggesting that signaling via IRS-2 was also impaired. Although the abundance of IRS-2 protein was similar in both groups, the altered electrophoretic mobility of IRS-2 in myotubes from women with PCOS suggested posttranslational modification of the protein, possibly degradation. This defect in IRS-2 signaling, if manifested in other tissues in vivo, could contribute to the metabolic (35) and the reproductive (7) PCOS phenotype.

Fig. 4. Tyrosine phosphorylation (PY) of insulin receptor (IR) and insulin receptor substrate (IRS)-1 in cultured myotubes of age- and BMI-matched obese women with PCOS and control women. A: basal autophosphorylation of IRβ in PCOS and control myotubes (n = 5/group) was assessed by ELISA. Tyrosine phosphorylation of IRβ (B) and IRS-1 (C) was determined in PCOS and control myotubes (n = 5/group) after incubation with insulin (100 nmol/l) for 10 min. Lysates (1 mg) were immunoprecipitated (IP) using anti-phosphotyrosine antibody followed by immunoblotting for IRβ or IRS-1. Tyrosine phosphorylation of IRS-1 was adjusted for IRS-1 protein content given higher IRS-1 abundance in myotubes from women with PCOS. Representative immunoblots for anti-IRβ (B) and anti-IRS-1 (C) are shown. Filled bars, myotubes from women with PCOS; open bars, control myotubes. Data are presented as means ± SE.

Fig. 5. Association of IRSs with p85 subunit of phosphatidylinositol (PI) 3-kinase and IRS-associated PI-3 kinase activity in cultured myotubes from age- and BMI-matched obese women with PCOS and control women (n = 6/group). Myotubes were treated with (+) or without (−) insulin (100 nmol/l) for 10 min. Anti-IRS-1 immunoprecipitates were resolved by SDS-PAGE (7.5% gels) and immunoblotted for p85 (A) and IRS-1 (not shown), and anti-IRS-2 immunoprecipitates for p85 (C) and IRS-2 (not shown). Representative p85 immunoblots are shown. PI 3-kinase activity [phosphorimager arbitrary units (AU) × 10^6] was measured in IRS-1 (B) and IRS-2 (D) immunoprecipitates from PCOS (P) and control (C) myotubes treated ± insulin for 10 min. IRS-1-associated PI 3-kinase results were adjusted for IRS-1 protein content, given higher IRS-1 abundance in myotubes from women with PCOS. Representative autoradiograms showing PI3P products are shown. Filled bars, myotubes from women with PCOS; open bars, control myotubes. Data are presented as means ± SE. *P < 0.05, **P < 0.01, paired t-test, PCOS vs control. #P = 0.05 Wilcoxon Signed-Rank test, PCOS vs control.
Glucose uptake and GLUT1 protein abundance were both increased by ~50% in myotubes from women with PCOS. This observation, taken together with the significant positive correlation between basal glucose uptake and GLUT1 protein levels, suggested that the increased abundance of this glucose transporter accounted for the change in glucose uptake in PCOS. The mechanisms and physiological relevance of this change are unknown, as GLUT1 does not appear to play an important role in glucose uptake in adult skeletal muscle (20). Its abundance increases as cultured skeletal muscle loses its differentiated phenotype (46); conversely, GLUT4 abundance increases as myoblasts fuse to form myotubes (3, 46). However, the increased abundance of GLUT1 in myotubes from women with PCOS could not be accounted for by a relative lack of differentiation, because other markers of differentiation were higher than those from control women, arguing against constitutive IR phosphorylation at IRS-1 Ser312, and insulin-stimulated IR tyrosine phosphorylation tended to be decreased in these cells. The reason for these differences in serine phosphorylation of insulin-signaling proteins in cultured skin fibroblasts compared with myotubes of women with PCOS may be related to tissue-specific differences in serine kinase expression or function (44). Tissue differences in insulin action on glucose metabolism and on lipolysis have been reported in PCOS (5, 9, 12, 18, 19) and T2DM (25, 43), consistent with tissue-specific regulation of insulin signaling.

In the cultured skeletal muscle of subjects with T2DM, significant decreases in glucose metabolism and insulin signaling persist (6, 22, 25, 26, 39, 41). Although the phenotype of mature skeletal muscle is not entirely reproduced in cultured muscle cells, these abnormalities reflect many of the major defects in vivo of subjects with T2DM (4, 10, 31, 33). There are also persistent defects in insulin action in myotubes from individuals with IGT that differ from those reported here, suggesting that such changes cannot be accounted for by the higher incidence of IGT in our subjects with PCOS (49). Thus our findings in PCOS differ from those reported in the aforementioned insulin-resistant conditions. With the caveat that these studies are not directly comparable because there may be variations in the experimental conditions, this observation, taken together with previous studies demonstrating distinctive defects in insulin action in adipocytes (9, 12), fibroblasts (14),...
and skeletal muscle (13) from women with PCOS, is consistent with our hypothesis that PCOS is a genetically unique disorder conferring an increased risk for T2DM (11, 12). It remains possible that hyperandrogenemia contributed to the distinctive PCOS phenotype, since in animal models transient prenatal or neonatal androgen exposure can permanently alter insulin action (17, 42). Although the physiological relevance of these changes is unknown, it is possible that they confer increased susceptibility to the insulin resistance-inducing effects of circulating factors such as FFA or TNF-α. It is clear that these changes represent a stable PCOS phenotype that persists in long-term culture and differs from findings in myotubes from control women well-matched for obesity, age, and ethnicity propagated in identical culture conditions.

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