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The adipose cell lineage is not intrinsically insulin resistant in polycystic ovary syndrome

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Abstract

Selective resistance to the effects of insulin on glucose metabolism in skeletal muscle and adipose tissue is a key feature of polycystic ovary syndrome (PCOS). The pathogenesis of insulin resistance in skeletal muscle in PCOS involves interaction of in vivo environmental factors with intrinsic defects in insulin signaling. We aimed to determine whether (1) intrinsic defects in insulin action/signaling and cytokine secretion were present in adipose cells in PCOS and (2) insulin resistance can be induced in control adipose cells by culture in medium conditioned by insulin-resistant PCOS fibroblasts. Subcutaneous abdominal preadipocytes from obese women with PCOS (n = 7) and age- and body mass index–matched controls (n = 5) were cultured for several generations in vitro. Basal and insulin-stimulated glycogen synthesis and basal glucose transport did not differ in the preadipocytes from women with PCOS and controls. Abundance of insulin receptor (IR) β subunit, insulin receptor substrate (IRS) 1 and 2, p85 subunit of phosphatidylinositol 3-kinase, and extracellular signal-regulated kinase (ERK)1/2 activation did not differ. Secretion of tumor necrosis factor α and interleukin 6 did not differ. Insulin action on glycogen synthesis in control preadipocytes was not altered by coculture with or growth in media conditioned by PCOS skin fibroblasts with constitutive serine phosphorylation of IRβ subunit (IR ser+), indicating that IR ser+ cells do not secrete an insulin resistance–inducing factor. We conclude that in contrast to skeletal muscle and skin fibroblasts, there is no evidence for intrinsic defects in insulin signaling in the PCOS adipose cell lineage, indicating that insulin resistance in these cells is likely due to factors in the in vivo environment.

1. Introduction

Polycystic ovary syndrome (PCOS) is among the most common endocrine disorders of premenopausal women. It is characterized by hyperandrogenism, disordered gonadotropin secretion, skeletal muscle insulin resistance, and an increased risk of type 2 diabetes mellitus (DM) [1-4]. Insulin resistance in PCOS is independent of obesity, but obesity plays an amplifying role [3-5]. Although adipocyte-derived factors are now known to play a key role in modulating insulin action in skeletal muscle [6], the adipose cell lineage in PCOS has not been comprehensively investigated. To date, studies using freshly isolated subcutaneous abdominal adipocytes from women with PCOS have demonstrated obesity-independent post–insulin receptor (IR)–binding resistance to the effects of insulin on glucose transport [7-10]. In addition, insulin inhibition of lipolysis is impaired in these cells [9,10], but not in adipocytes isolated from the visceral depot [11].

Recent studies of skeletal muscle in PCOS have provided evidence for the interaction of intrinsic defects in insulin signaling with factors in the in vivo environment in the pathogenesis of insulin resistance in this tissue. Impaired insulin-stimulated glucose uptake in skeletal muscle in vivo of women with PCOS is associated with decreased insulin receptor substrate (IRS) 1–associated phosphatidylinositol 3-kinase (PI3K) activation [12]. However, when skeletal muscle cells from women with PCOS are grown in culture, insulin-stimulated glucose uptake is not impaired despite defects in insulin signaling via both IRS-1 and IRS-2, indicating that in vivo factors are required for insulin resistance to be manifested [13]. The persistence of these signaling defects in cells cultured for

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generations outside their in vivo environment suggests that the defects are intrinsic, although whether these changes are genetic or epigenetic remains to be elucidated. Impaired insulin signaling in skeletal muscle in PCOS is selective for glucose metabolic pathways: mitogenic signaling via extracellular signal-regulated kinase (ERK)1/2 is enhanced [14]. When skin fibroblasts from women with PCOS are cultured for multiple generations, these cells also display insulin resistance selective for metabolic pathways [15] due to a distinct molecular mechanism. Insulin receptors purified from these fibroblasts show constitutive serine phosphorylation (IR ser+) with inhibition of insulin-stimulated tyrosine phosphorylation caused by a serine kinase extrinsic to the receptor [16,17].

The potential roles of environmental factors and intrinsic insulin signaling defects in the pathogenesis of insulin resistance have not been evaluated in the adipose cells of women with PCOS. The aim of this study was to determine whether intrinsic defects in insulin signaling were present in PCOS adipose cells by assessing insulin action in cells grown for multiple generations removed from the influences of the in vivo environment. Preadipocytes, the fibroblast-like precursor cells present in the stromal fraction of adipose tissue, differentiate in vivo into mature lipid-laden adipocytes [18]. Although primary cultures of human preadipocytes show a high capacity for differentiation to adipocytes in vitro, this differentiation capacity is lost once preadipocytes have been allowed to divide for only a few generations [19]. We therefore elected to study insulin action in the cultured preadipocytes of women with PCOS and age- and body mass index (BMI)–matched controls. We hypothesized that intrinsic defects would be present in cultured preadipocytes from women with PCOS, similar to our findings in cultured skin fibroblasts [15] and skeletal muscle [13,14]. Given that proinflammatory cytokines have been implicated in impairment of insulin signaling [20-25], we also measured secretion of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) by preadipocyte cultures of women with PCOS and controls. Finally, we carried out experiments using control preadipocyte cultures to determine if IR ser+ fibroblasts secreted an insulin resistance–inducing factor.

2. Subjects, materials, and methods

2.1. Subjects

The study was approved by the Institutional Review Board at Brigham and Women's Hospital, Boston, MA, and all subjects gave written, informed consent. Seven obese women with PCOS and 5 age-, BMI- and ethnicity-matched control women were studied. Women were aged 21 to 40 years, in good health, 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 3 months before the study. Control women had regular menstrual cycles (every 27-35 days), no clinical or biochemical evidence of hyperandrogenism, and no first-degree relatives with type 1 or 2 DM. Women with PCOS had irregular menstrual cycles ($\leq$6 menses per year) and elevated total testosterone and/or non–sex hormone–binding globulin-bound testosterone levels [1,5,7,16]. Nonclassical congenital adrenal hyperplasia due to 21-hydroxylase deficiency, androgen-secreting neoplasms, and hyperprolactinemia were excluded by appropriate tests in the women with PCOS. All subjects underwent a 75-g oral glucose tolerance test after an overnight fast, with glucose and insulin levels measured basally and 2 hours post glucose load. World Health Organization criteria were used to assess glucose tolerance [26]. Fasting glucose levels were also assessed by using American Diabetes Association criteria [27].

2.2. Harvesting and culture of preadipocytes

Subjects underwent aspiration of subcutaneous abdominal adipose tissue under local anesthesia after an overnight fast as previously described [7]. The stromal cell fraction was obtained by collagenase digestion and centrifugation using the method of Rodbell [28] and cultured to near confluence in low-glucose Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL Life Technologies), 50 IU/mL penicillin, and 50 μg/mL streptomycin (Cellgro, Mediatech, Herndon, VA). All experiments were performed on subcultured cells (passage 2) grown at a density of 3000 cells/mL until near confluence (total population doublings, 11 ± 1, mean ± SD) and incubated in minimum essential medium alpha (αMEM; Gibco BRL Life Technologies) supplemented with 0.1% bovine serum albumin (BSA fraction V; Boehringer Mannheim, Indianapolis, IN) for 2 hours before assays or lysis.

2.3. Glycogen synthesis and glucose transport assays

Glycogen synthesis was determined by measuring glucose incorporation into glycogen in duplicate wells for each condition by using the method of Henry et al [29] with the modification that cultures were washed with serum-free medium and then incubated in the same medium with or without insulin (human recombinant regular insulin, Novo Nordisk, Princeton, NJ) plus D-[U-^{14}C]glucose (NEN Life Science Products, Boston, MA) for 2 hours. Lysates from 2 wells per 6-well dish were assayed for protein concentration (Bio-Rad Laboratories, Hercules, CA). Glucose uptake in 6 replicate wells for each condition was measured by using the method of Ciaraldi et al [30], under conditions where cell membrane glucose transport was rate limiting. An aliquot of lysate from each well was assayed for protein concentration and the remainder was counted by using liquid scintillation counting. Specific glucose transport was calculated by subtracting L-[^{14}C]glucose incorporation from [^{3}H]deoxy-

D-glucose (NEN Life Science Products) incorporation in each well.

2.4. Western blotting

Confluent preadipocytes were incubated in αMEM + 0.1% BSA for 2 hours followed by incubation in the same medium with or without insulin (100 nmol/L) for 10 minutes, then lysed as reported [16]. Western blotting was carried out as previously described [12], with PCOS and control samples run together on the same gels. Membranes were probed with antibodies to IRβ subunit (Transduction Laboratories, San Diego, CA), IRS-1, IRS-2 (a gift from Dr M. White, Joslin Diabetes Center, Boston, MA), p85 subunit of PI3K (Upstate Biotechnology, Lake Placid, NY), ERK1/2 or phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA), then incubated with appropriate secondary antibodies (Bio-Rad Laboratories). The products were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) and quantitated by using a scanning densitometer (Bio-Rad Laboratories). The same internal standard was run in all immunoblots for each protein and results are expressed as percentage of internal standard.

2.5. Secretion of TNF-α and IL-6

Confluent preadipocytes were incubated in αMEM + 0.1% BSA for 12 hours, then the media were replaced with fresh αMEM + 0.1% BSA. After 48 hours, the media were collected and aliquots were assayed in duplicate for IL-6 and TNF-α using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Quantikine HS, R&D Systems, Minneapolis, MN).

2.6. Conditioned media and coculture experiments

The PCOS IR ser+ skin fibroblasts used in both conditioned media and coculture experiments had previously been shown to belong to a subpopulation of PCOS fibroblasts with significantly increased baseline IRβ serine phosphorylation with decreased insulin-stimulated tyrosine phosphorylation [16]. Insulin-stimulated glycogen synthesis was measured in control preadipocytes cultured in medium that had been conditioned by prior incubation with PCOS IR ser+ or control skin fibroblast cultures. Fresh growth medium (15% vol/vol) was added to the conditioned media and media were changed twice weekly until near confluence. In addition, insulin-stimulated glycogen synthesis was measured in control preadipocytes cocultured with PCOS IR ser+ or control fibroblasts by using dual-chamber culture dishes (Becton Dickinson, Franklin Lakes, NJ). Duplicate wells were assayed for each condition.

2.7. Statistical analysis

Data were analyzed by Student t test for comparison between (unpaired) and within (paired) groups. Data were log transformed as necessary to achieve homogeneity of variance. Data are presented as mean ± SEM with significance at $P < .05$.

3. Results

3.1. Subject characteristics

By design, the PCOS and control subjects were comparable for age and BMI (Table 1). Compared with control women, women with PCOS had significant elevations of serum total and biologically available testosterone. No subject had abnormal fasting glucose levels but 2 subjects with PCOS had impaired glucose tolerance and 1 subject with PCOS fulfilled criteria for DM by World Health Organization criteria. Control subjects had normal fasting and post–glucose load plasma glucose levels. Mean fasting glucose was significantly higher in the PCOS subjects ($P < .05$) and also tended to be higher at 2 hours after glucose load ($P = .06$). Plasma insulin 2 hours after 75-g glucose load was significantly elevated in women with PCOS compared with controls ($P < .01$), consistent with the presence of insulin resistance.

3.2. Glucose transport and glycogen synthesis

Basal glucose transport did not differ in cultured preadipocytes from women with PCOS and controls (Fig. 1A). There was no stimulation of glucose transport by insulin (data not shown), in keeping with the absence of glucose transporter 4 in preadipocytes [31]. However, glucose incorporation into glycogen was stimulated by a mean of 2.4-fold (range, 1.5-3.5) in both groups, probably through activation of glycogen synthase [32]. Glucose incorporation into glycogen did not differ in cultures from women with PCOS and controls at baseline or after insulin stimulation (Fig. 1B).

3.3. Abundance of proximal insulin signaling proteins

The baseline abundance of IRβ (Fig. 2A), IRS-1 (Fig. 2B), p85 subunit of PI3K (Fig. 2C), and IRS-2

Table 1
Subject characteristics

	Control	PCOS
No. of subjects	5	7
Age (y)	30 ± 3	29 ± 2
BMI (kg/m^2)	37.5 ± 2.9	38.8 ± 1
Total testosterone (nmol/L)	0.8 ± 0.1	2.6 ± 0.3***
Unbound testosterone (nmol/L)	0.2 ± 0.1	0.9 ± 0.1***
DHEAS (μmol/L)	4.8 ± 0.8	5.8 ± 0.8
Glucose (mmol/L), fasting	4.4 ± 0.2	5.2 ± 0.2*
Glucose (mmol/L), 2 h after 75-g glucose	5.9 ± 0.3	8.1 ± 0.8
Insulin (pmol/L), fasting	100 ± 21	186 ± 43
Insulin (pmol/L), 2 h after 75-g glucose	387 ± 29	1134 ± 316**

Data are mean ± SEM. DHEAS indicates dehydroepiandrosterone.
* $P < .05$, unpaired t test.
** $P < .01$, unpaired t test.
*** $P < .0001$, unpaired t test.

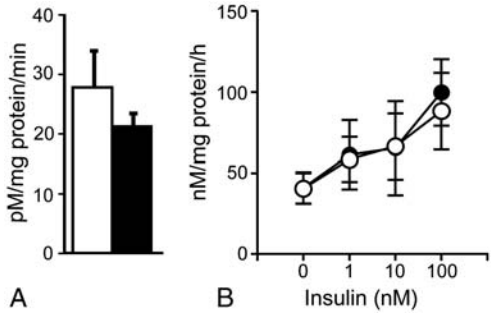

Fig. 1. Basal glucose uptake and insulin-stimulated glycogen synthesis in cultured preadipocytes of obese women with PCOS and age- and BMI-matched controls. A, Glucose uptake (n = 7 PCOS, n = 5 controls) was assessed at baseline. B, Glycogen synthesis (n = 6 PCOS, n = 5 controls) was measured at baseline and after incubation with insulin (1-100 nmol/L). Filled circles represent PCOS and open circles represent controls. Filled bars represent PCOS and open bars represent controls. Data are presented as mean ± SEM.

(Fig. 2D) did not differ in preadipocytes from women with PCOS and controls.

3.4. ERK1/2 expression and activation

Phospho-ERK1/2 abundance did not differ at baseline or after insulin stimulation in preadipocytes from women with PCOS and controls (Fig. 3A). Stimulation of ERK1/2 phosphorylation with insulin, mean 1.3-fold, did not reach significance compared with baseline in either group. The abundance of total ERK1/2 protein did not differ in PCOS and control groups (Fig. 3B).

3.5. Secretion of TNF-α and IL-6

Media concentrations of TNF-α and IL-6 did not differ significantly in cultured preadipocytes from women with

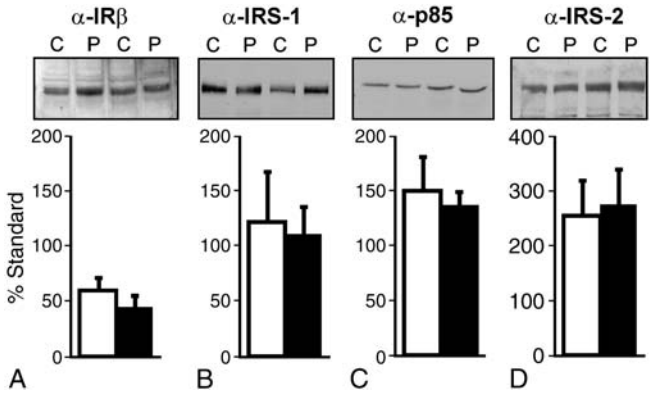

Fig. 2. Abundance of proximal insulin signaling proteins in cultured preadipocytes from obese women with PCOS and age- and BMI-matched controls. Abundance of IRβ (A), IRS-1 (B), p85 subunit of PI3K (C), and IRS-2 (D) (n = 6 PCOS, n = 5 controls) determined by immunoblot analysis of preadipocytes from women with PCOS (P) and controls (C). Basal lysates (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% gels) and representative immunoblots are shown. Filled bars represent PCOS and open bars represent controls. Data are presented as mean ± SEM.

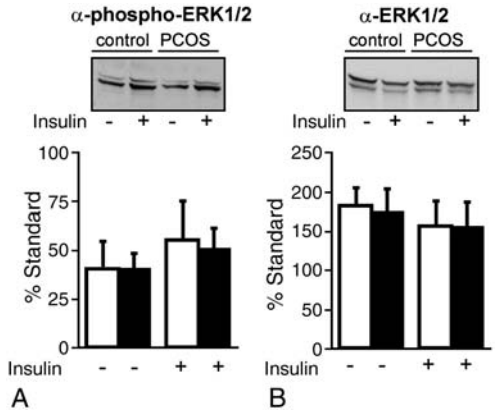

Fig. 3. Phospho- and total ERK1/2 in cultured preadipocytes from obese women with PCOS and age- and BMI-matched controls. Cultured preadipocytes (n = 6 PCOS, n = 5 controls) were incubated with (+) or without (−) insulin (100 nmol/L) for 10 minutes and lysates (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% gels). Blots were probed with anti–phospho-ERK1/2 (A), then stripped and reprobed with anti–total ERK1/2 (B). Representative immunoblots are shown. Filled bars represent PCOS and open bars represent controls. Data are presented as mean ± SEM.

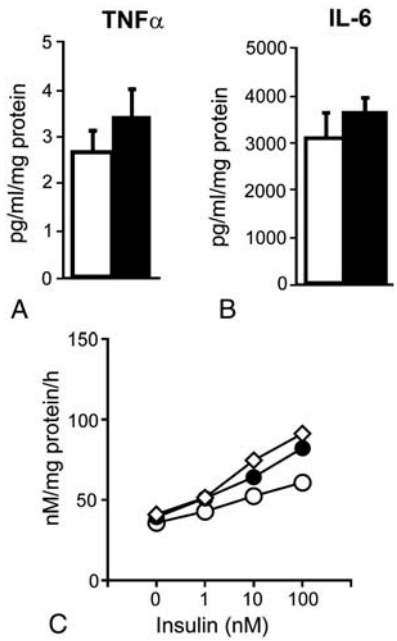

Fig. 4. Secretion of cytokines by cultured preadipocytes from obese women with PCOS and age- and BMI-matched controls. Serum-free media from confluent preadipocytes (n = 7 PCOS, n = 5 controls) were collected after 48 hours' incubation and assayed for TNF-α (A) and IL-6 (B) using enzyme-linked immunosorbent assay. Filled bars represent PCOS and open bars represent controls. Results are expressed as picograms per milliliter of media and adjusted for protein content of each dish. Insulin-stimulated glycogen synthesis was measured in control preadipocytes cultured with media that had been conditioned by control cultured skin fibroblasts (open circles), by PCOS fibroblasts with constitutive increased serine phosphorylation of IRβ (IR ser+) (filled circles), or nonconditioned media (open diamonds) (C). Data are presented as mean ± SEM.

PCOS and controls, whether expressed as picograms per milliliter per milligram protein (TNF-α: PCOS, 3.4 ± 0.6; control, 2.7 ± 0.4; IL-6: PCOS, 3595 ± 340; control, 3098 ± 533) (Fig. 4A, B) or picograms per milliliter per million cells (TNF-α: PCOS, 1.7 ± 0.4; control, 1.1 ± 0.2; IL-6: PCOS, 891 ± 285; control, 1068 ± 262). A sample of approximately 29 subjects with PCOS and 10 controls would have been needed for the observed differences in TNF-α secretion (picograms per milliliter per million cells) to achieve statistical significance at $P < .05$.

3.6. Media conditioning and coculture with PCOS IR ser+ fibroblasts

Basal and insulin-stimulated glycogen synthesis in control preadipocytes were not significantly changed by culturing in media conditioned by PCOS IR ser+ skin fibroblasts (Fig. 4C). Similarly, coculture with PCOS IR ser+ skin fibroblasts did not alter glycogen synthesis in control preadipocytes (data not shown).

4. Discussion

The present study was carried out to investigate whether the impairment in insulin action observed in vivo in adipocytes from women with PCOS results from intrinsic or acquired defects. Although it was not possible to assess glucose transport [31], insulin-stimulated glycogen synthesis was not impaired in cultured preadipocytes from women with PCOS, in contrast with previous studies of cultured PCOS skin fibroblasts [15]. The abundance of key proteins of the proximal insulin signaling pathway did not differ in PCOS and controls. These findings, in cells grown for many generations outside their in vivo environment, suggest that the defects in glucose metabolism observed in adipose cells in vivo of women with PCOS are acquired secondary to the hormonal/metabolic milieu rather than due to intrinsic defects. Ideally, the current experiments would have used preadipocytes cultured for several generations and then terminally differentiated in vitro to produce mature adipocytes, similar to the approach used in our previous studies of skeletal muscle in PCOS [13,14]. This approach was not feasible, as preadipocytes that have been allowed to divide for several generations lose their capacity to differentiate [19]. Although it is theoretically possible that intrinsic defects in insulin action in the adipocyte lineage may be manifested only in fully differentiated adipose cells, studies of the other major insulin-target tissue, skeletal muscle, have shown defects to be present in undifferentiated cells (myoblasts) as well as in mature terminally differentiated cells (myotubes) [33-36].

In contrast to the findings in preadipocytes, our studies in skeletal muscle have shown that both intrinsic defects in insulin signaling and factors in the in vivo environment contribute to the development of insulin resistance in this tissue in women with PCOS. In PCOS, both in vivo and in cultured skeletal muscle cells, IRS-1–associated PI3K activity was reduced [12,13]. However, in contrast to skeletal muscle in vivo, insulin-stimulated glucose uptake was not impaired in cultured skeletal muscle of women with PCOS, associated with a compensatory increase in IRS-1 protein abundance [13]. Several additional defects were present in the cultured skeletal muscle cells in PCOS, consistent with intrinsic defects. These included defects in signaling via IRS-2, abnormal electrophoretic mobility of IRS-2 suggestive of partial degradation, and increased non–insulin-mediated glucose transport associated with increased glucose transporter 1 protein abundance [13]. In addition, we have also reported enhanced ERK1/2 phosphorylation in skeletal muscle in vivo and in vitro of women with PCOS, an abnormality that may contribute to insulin resistance through phosphorylation of IRS-1 at the critical Ser^{312} site [14]. Although the molecular basis for these abnormalities in skeletal muscle in PCOS has not yet been determined, it is clear that they represent tissue-specific defects, as they are not present in preadipocytes in PCOS.

Several adipose cell products are candidate factors for the induction of insulin resistance in skeletal muscle. These include free fatty acids (FFA), which are thought to inhibit insulin signaling by activating protein kinase C isoforms that serine phosphorylate IRS-1 [37]. Tumor necrosis factor α also inhibits insulin signaling through serine phosphorylation of IRS-1 [20]. Other proinflammatory cytokines have been implicated in the pathogenesis of insulin resistance, including IL-6 [25]. In women with PCOS, circulating TNF-α levels have not consistently been found to be elevated [38,39], although mononuclear cells from obese women with PCOS show enhanced TNF-α release in response to hyperglycemia compared with controls [40]. Likewise, circulating IL-6 levels do not differ in women with PCOS and age- and BMI-matched controls [41,42]. Adipose cell products may also act locally by paracrine/autocrine mechanisms to induce insulin resistance. Adipose tissue expression of TNF and IL-6 is higher in insulin-resistant subjects [23], and adipose tissue IL-6 content correlates with impairment of insulin action in adipocytes in obese subjects with and without DM [24]. In the current study, there were no differences in production of TNF-α or IL-6 by preadipocytes in vitro in PCOS compared with controls. However, the study was underpowered to detect a significant difference in TNF-α production.

We also investigated the possibility that cultured PCOS skin fibroblasts with constitutive increase in serine phosphorylation of IR (IR ser+) could secrete an insulin resistance–inducing factor. Cultured human skin fibroblasts have the capacity to synthesize several growth factors and cytokines [43] including IL-6 [44]. However, neither growth in IR ser+ conditioned media nor coculture with IR ser+ fibroblasts altered insulin responsiveness in control preadipocytes, indicating that factors secreted by PCOS skin fibroblasts that act via a paracrine mechanism are not implicated in the selective insulin resistance of these cells [15].

In conclusion, the present studies have provided evidence that impaired adipocyte insulin responsiveness in vivo in PCOS is acquired due to factors in the in vivo environment rather than due to intrinsic defects in insulin signaling. The abnormalities we observed in cultured skeletal muscle in PCOS including elevated non–insulin-mediated glucose uptake and enhanced activation of ERK1/2 were not present in preadipocytes. In addition, preadipocytes from women with PCOS did not demonstrate resistance to insulin's effects on glycogen synthesis as found in cultured skin fibroblasts, indicating important tissue-specific differences in this syndrome.

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