A Unique Defect in the Regulation of Visceral Fat Cell Lipolysis in the Polycystic Ovary Syndrome as an Early Link to Insulin Resistance

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The etiology of polycystic ovary syndrome (PCOS) is unknown. However, PCOS has a strong resemblance to the insulin resistance (metabolic) syndrome, where an increased rate of visceral fat cell lipolysis is believed to play a pathophysiological role. We hypothesized that primary defects in visceral lipolysis might also exist in PCOS. Ten young, nonobese, and otherwise healthy PCOS women were compared with 13 matched control women. In vitro lipolysis regulation and stoichiometric properties of the final step in lipolysis activation, namely the protein kinase A (PKA)-hormone sensitive lipase (HSL) complex, were investigated in isolated visceral (i.e., omental) fat cells. Body fat distribution and circulating levels of insulin, glucose, and lipids were normal in PCOS women. However, in vivo insulin sensitivity was slightly decreased ($P = 0.03$). Catecholamine-induced adipocyte lipolysis was markedly (i.e., about twofold) increased in PCOS women due to changes at the postreceptor level, although there was no change in the antilipolytic properties of visceral fat cells. Western blot analyses of visceral adipose tissue showed twofold increased levels of the catalytic and the regulatory Iα components of PKA. In contrast, the regulatory RIIβ component of PKA was almost 50% decreased in visceral adipose tissue in PCOS women. Recent studies on genetically modified mice have shown that a similar transition in the regulatory PKA units induces an increased lipolytic response to catecholamines. Further analysis showed that the level of HSL-short, an enzymatically inactive splice form of HSL, was decreased in PCOS ($P < 0.01$). The altered lipolysis in PCOS is different from that observed in visceral fat cells in the insulin resistance syndrome that occurs at the level of adrenergic receptors. We concluded that increased catecholamine-induced lipolysis in visceral fat cells may be due to unique alterations in the stoichiometric properties of the adipose PKA-HSL holoenzymes. This could be an early and possibly primary lipolysis defect in PCOS.
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Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, affecting up to 10% of women in reproductive age (1). It is not only the most frequent cause of anovulation, hirsutism, and hyperandrogenism but is also closely linked to insulin resistance, hyperinsulinemia, glucose intolerance, dyslipidemia, and type 2 diabetes (1–3). The onset of clinical manifestation in PCOS often occurs at the time of puberty (4). Though obesity is common, it is not universal. The primary abnormality in PCOS remains to be established despite the fact that it has been proposed to be of central, ovarian, adrenal, or peripheral metabolic origin (3). Though insulin resistance is found in lean as well as obese PCOS patients (5), obesity and PCOS may exert independent effects on insulin resistance. Weight loss, which reduces insulin resistance, improves some but not all of the endocrine and metabolic disturbances. Also, drugs that improve insulin sensitivity have reduced hyperandrogenism in PCOS (6).

There are close points of similarity between PCOS and the so-called insulin resistance (metabolic) syndrome (7,8). In the latter syndrome, disturbances in fatty acid release from visceral adipose tissue through alterations in visceral adipocyte lipolysis are believed to play a major pathophysiological role (9,10). Visceral adipose tissue has a unique anatomy due to being drained by the portal vein. A high level of "portal" fatty acids will directly affect the liver and cause hyperinsulinemia, dyslipidemia, glucose intolerance, and insulin resistance. Human fat cell lipolysis is under hormonal regulation by insulin (inhibition) and catecholamines (stimulation) and also by inhibitory parahormones such as prostaglandins and adenosine. In the metabolic syndrome, it was found that catecholamine-induced lipolysis was accelerated in visceral fat cells due to adrenergic receptor alterations, such as increased activity of the lipolytic β_3-receptor and decreased activity of the antilipolytic α_2-receptor (11,12).

In the insulin resistance syndrome, catecholamine action in subcutaneous fat cells, in contrast to visceral fat cells, is decreased due to a combination of decreased β_2-adrenergic expression and decreased function of the protein kinase A (PKA)-hormone sensitive lipase (HSL)

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8bcAMP, 8-bromocyclic AMP; CT, computer tomography; dcAMP, dibutyryl cAMP; EC_{50}, half-maximum effect; HSL, hormone sensitive lipase; OD, optical density; PCOS, polycystic ovary syndrome; PKA, protein kinase A; SHBG, sex hormone–binding globulin; WHR, waist-to-hip ratio.

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complex (13). An almost identical defect, which is not attributed to hyperandrogenism (14,15), is observed in lean women with PCOS. It is not known whether defects in visceral lipolysis occur in PCOS.

There are no ideal animal models of PCOS (16). Consequently, the present study was designed to investigate the lipolysis regulation of visceral adipose tissue in patients with PCOS. We examined young, lean PCOS women who were otherwise healthy in order to identify a possible primary defect.

RESEARCH DESIGN AND METHODS

Patients and experimental protocol. Ten nonobese women with PCOS and 13 healthy BMI-matched women participated in the study. For all subjects, age ranged from 24 to 39 years and BMI from 19 to 27 kg/m^2. The women with PCOS were recruited among those undergoing laparoscopic ovarian cautery in the infertility unit at the hospital. The diagnosis was based on clinical findings of hyperandrogenism of ovarian origin, menstrual disturbances, oligomenorrhea, or amenorrhea indicating anovulation, increased leutinizing hormone/follicle-stimulating hormone ratio, and a typical appearance of the ovaries on transvaginal ultrasound. All the ultrasound examinations were performed by one of the authors (I.E.). The control subjects were recruited from the waiting list for laparoscopic sterilization operation. Patients and control subjects were not on medication. None of the subjects were either completely sedentary or involved in athletic performance. All had standard dietary habits, and none had been on a slimming diet during the year preceding the study. One PCOS woman and one control subject were smokers, with a consumption of less than five cigarettes a day. Both quit smoking 4 weeks before all examinations. All other subjects were nonsmokers. Within 14 days before the operation, the women were examined at 8:00 A.M. after an overnight fast. Waist-to-hip ratio (WHR), BMI, and blood pressure were determined. After 30 min of rest in the supine position, a serum sample was obtained for analysis of hormones, sex hormone–binding globulin (SHBG), and glucose at the hospital's clinical chemistry laboratory. The insulin tolerance test (K_{itt}) was performed in succession, as previously described in detail (17). This test is a measure of overall insulin sensitivity and shows a high degree of correlation with the "golden standard" euglycemic-hyperinsulinemic clamp test (18). Subcutaneous and visceral adipose tissue area correlating to total and adipose tissue area were determined by computer tomography (CT) at the L3 and L4 level, as previously described (19). The ratio between testosterone and SHBG was used as an index of biologically active testosterone (20). General anesthesia using propofol in combination with fentanyl and midazolam was induced at 8:00 A.M. after an overnight fast and maintained by propofol and a mixture of oxygen and nitrous oxide. Muscle relaxation was induced and maintained by suxamethonium chloride. Intravenous saline was administered before the fat biopsies (~1 g), which were taken from the major omentum at the beginning of the operation. The committee on ethics at the Karolinska Institute approved the study. The study protocol was explained in detail to the participants, and their consent was obtained.

Isolation of fat cells and determination of fat cell size and number. One piece, ~300 mg, was frozen in liquid nitrogen and stored at −70°C for subsequent Western blot analysis. Isolated fat cells were prepared, as described by Rodbell (21). Direct microscopic determination of the fat cell diameter was performed as previously described (20). The mean fat cell volume and weight were determined as previously described (22). The total lipid content of the aliquot was determined gravimetrically after organic extraction, and the number of fat cells could be calculated by dividing the total lipid weight by the mean cell weight. This method gives the same results as a direct method to determine cell number (23,24).

Lipolysis experiments. Isolated fat cells were incubated as previously described in detail (11–15). In brief, diluted suspension of fat cells were incubated in duplicate for 2 h with or without increasing concentrations of either norepinephrine, the nonselective β-adrenergic agonist isoprenaline, the selective β_1-adrenoceptor agonist dobutamine, the selective β_2-adrenoceptor agonist terbutaline, the selective partial β_3-adrenoceptor agonist CGP 12177, the phosphodiesterase-resistant cAMP analog dibutyryl cAMP (dcAMP), the selective α_2-adrenoceptor agonist UK 14304, phenylisopropyl adenosine (a nonmetabolizing adenosine analog), prostaglandin E_2, or insulin. All incubations were performed at 37°C in Krebs-Henseleit phosphate buffer (pH 7.4), supplemented with glucose (1 g/l), BSA (20 g/l), and ascorbic acid (0.1 g/l) and, in the antilipolytic experiments, adenosine deaminase (1 mU/ml) as well as 10^{-3} mmol/l of 8-bromocyclic AMP (8bcAMP), with air as the gas phase. After incubation, a cell-free aliquot was removed for determination of glycerol, which is an index of lipolysis.

The concentration (log mol/l) of agonist causing half-maximum effect (EC_{50}) was determined using logistic conversion of each concentration response curve. The maximum effect (responsiveness) of each agonist was determined as glycerol release at the maximum effective agonist concentration.

Protein isolation and Western blot analysis. Frozen tissue, ~300 mg, was crushed and lysed in protein lysis buffer (1% Triton-X100, Tris-HCl pH 7.6, and 150 mmol/l NaCl, 4°C), supplemented with protease inhibitors (1 mmol/l PMSF [phenylmethylsulfonyl fluoride] and Complete; Boehringer Mannheim, Mannheim, Germany), and homogenized using a microtome. The homogenate was centrifuged at 14,000 rpm for 30 min, and the infranatant was removed and saved. The protein content in each sample was determined using a kit of reagents from Pierce Biotech (Rockford, IL). One hundred micrograms of total protein was then loaded on polyacrylamide gels and separated by standard SDS-PAGE. Samples from PCOS and control subjects were run on the same gels and transferred to the same PVDF (polyvinylidine fluoride) membranes (Amersham Pharmacia Biotech, Little Chafford, U.K.). Blots were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20 and 5% nonfat dried milk. This was followed by an overnight incubation at 4°C in the presence of antibodies directed against either the HSL complex, the catalytic region of PKA (PKAcat, 1:1,000), the regulatory regions of PKA Iα and IIβ (PKAreg Iα and PKAreg IIβ, 1:1,000), and finally against all subforms of regulatory region I (PKAreg I, 1:1,000). All primary antibodies, except those against HSL, were from Transduction Laboratories (Lexington, KY). To confirm antibody specificity, positive controls were included in all experiments as provided by the manufacturer. HSL antibodies were generated by one of the authors (C.H.), as described below. Secondary antibodies conjugated to horseradish peroxidase were from Sigma (St. Louis, MO) (α-mouse 1:5,000, α-rabbit 1:4,000, and α-chicken 1:2,500). Antigen-antibody complexes were detected by chemiluminescences using a kit of reagents from Pierre (Supersignal; Rockford, Rockford, IL), and blots were exposed to high-performance chemiluminescence film (Amersham, Little Chalfont, UK). Films were scanned, and the optical density (OD) of each specific band was analyzed using the Image program (National Institutes of Health, Bethesda, MD) and expressed as OD · mm^{-2} · 100 μg^{-1} of total protein.

HSL antibodies. Human HSL exists in two forms due to alternative splicing of exon 6 (25). These are commonly referred to as HSL-long and -short. In the present study, we used an antibody that is specific for the long variant and another that recognizes both forms. The HSL-long antibody was generated in rabbits, as previously described (25). A 15-residue synthethic peptide (QPAASPSRLLSLMDBP), derived from the amino acid sequence encoded by exon 6, was coupled to keyhole limpet hemocyanin via an added COOH-terminal cystein residue and was used to immunize the rabbits. The antiserum was shown to specifically recognize full-length human HSL (HSL-long), whereas it did not recognize the splice variant lacking exon 6 (HSL-short). Antibodies recognizing both HSL-long and -short were generated in chicken as previously described (26,27). In short, immunized chicken antiserum was affinity-purified against recombinant rat HSL coupled to a CNBr-activate Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). The affinity-purified antibodies were shown to be specific for both forms of HSL.

Drugs and chemicals. BSA (fraction V, A4503), *Clostridium histolyticum* collagenase type 1, glycerol kinase from *Escherichia coli* (C-0130), forskolin (Calbiochem 344270; Calbiochem, La Jolla, CA), and norepinephrine [(−)-Arterenol A-9512] dcAMP were obtained from Sigma Chemical (St. Louis, MO). Adenosine deaminase ADA was of calf intestine origin and was supplied by Roche Diagnostics Scandinavia (Bromma, Sweden). Dobutamine hydrochloride was purchased from Eli Lilly (Indianapolis, IN), and terbutaline sulfate was obtained from Astra (Lund, Sweden). CGP (±)12177 [(−)-4-(3-*t*-butylamino-2-hydroxy-propoxy)-benzimidazole-2-one] was from Ciba Geigy (Basel), and UK 14304 tartrate was from Pfizer (Sandwich, U.K.). ATP monitoring reagent containing firefly luciferase came from Biothema (Stockholm). All other chemicals were of the highest grade of purity commercially available.

Statistics. Student's two-tailed *t* test was used for comparison of data between (unpaired) and within (paired) groups. Data were also analyzed by ANOVA, taking into account age or fat cell volume by covariance analysis and drug concentration by repeated measure analysis. The SD was used as a measure of dispersion of clinical characteristics data (Table 1), and the SE was used for experimental data when normally distributed. Linear regression analysis was also performed. All statistical calculations were performed with a statistical software package (Statistica; StatSoft, Tulsa, OK).

RESULTS

Clinical characteristics. Clinical data are shown in Table 1. The women with PCOS had a slight but significant

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TABLE 1
Clinical characteristics

	PCOS	Control	*P*
n	10	13	NS
Age (years)	29 ± 3	33 ± 3	0.001
BMI (kg/m^2)	23.1 ± 3.2	23.8 ± 2.9	NS
WHR	0.81 ± 0.07	0.76 ± 0.05	NS
FS-FSH (IU/l)	4.7 ± 2.6	3.5 ± 1.6	NS
FS-LH (IU/l)	11.1 ± 5.4	3.2 ± 2.5	0.004
FS-LH/FS-FSH	2.8 ± 1.6	1.2 ± 1.3	0.02
FS-DHEAS (umol/l)	6.0 ± 4.0	3.3 ± 1.4	NS
FS-Prolactin (μg/l)	12.7 ± 6.3	9.8 ± 5.8	NS
FS-GH (μg/l)	3.2 ± 7.7	4.3 ± 4.4	NS
FS-Cortisol	326 ± 122	316 ± 67	NS
FS-Testosterone (nmol/l)	2.5 ± 2.0	1.2 ± 0.3	NS
FS-SHBG	36 ± 16	67 ± 28	0.01
T/SHBG	0.070 ± 0.041	0.022 ± 0.012	0.004
FS-17-OPH (nmol/l)	4.2 ± 2.9	2.2 ± 1.5	NS
FP-Cholesterol (mmol/l)	5.1 ± 0.9	4.6 ± 0.5	NS
FP-HDL cholesterol (mmol/l)	1.3 ± 0.4	1.6 ± 0.3	NS
FP-Triglycerides (mmol/l)	1.4 ± 1.5	0.8 ± 0.2	NS
FS-Insulin (mU/l)	6.95 ± 2.17	6.27 ± 0.96	NS
FB-Glucose (mmol/l)	4.76 ± 0.22	4.82 ± 0.42	NS
K_{itt} (4–16 min)	4.71 ± 0.87	5.50 ± 0.59	0.03
% Visceral fat	26 ± 9	25 ± 9	NS
% Subcutaneous fat	71 ± 9	72 ± 9	NS
Fat cell volume (pl)	211 ± 112	168 ± 91	NS

All values are means ± SD. Groups were compared using Student's unpaired two-tailed *t* test. FSH, follicle-stimulating hormone; LH, leutinizing hormone; K_{itt} (4–16 min) = % reduction of glucose concentration per minute in the insulin tolerance test (ITT); NS, not significant. DHEAS, dihydroepiandeterone; FB, fasting blood; FP, fasting plasma; FS, fasting serum; OPH, alpha hydroxiprogesterone; T, testosterone.

($P = 0.03$) decrease in K_{itt} indicative of some in vivo insulin resistance. However, they showed no other features of the insulin resistance syndrome because WHR, fasting plasma levels of insulin, glucose, triglycerides, and cholesterol level, as well as BMI, were comparable with those of control women. There was no difference between PCOS patients and the control subjects concerning the estimated subcutaneous and visceral adipose tissue, irrespective of whether the total area or the proportion of the different fat depots was determined by CT at the L3 and L4 levels (values not shown for L4). Likewise, there was no difference in fat cell weight between the groups. However, PCOS women were on average 4 years younger than control women ($P = 0.01$). Blood pressure was normal in both groups (values not shown). As expected from measurements of sex hormones and binding proteins, the PCOS women had hyperandrogenemia.

Lipolysis experiment. The mean concentration response curves for agonist-induced lipolysis are shown in Figs. 1–3. Stimulation of lipolysis with norepinephrine or β_1-, β_2-, and β_3-adrenoceptor selective agonists was much more efficient in PCOS than in control subjects. This was also true for stimulation at the level of adenylyl cyclase with forskolin or at the PKA level with dcAMP. All mean curves for lipolysis-stimulating agents differed significantly (*P* from 0.02 to 0.04) when PCOS and the control state were compared. However, inhibition of lipolysis at the level of insulin receptor, α_2-adrenoceptor, as well as at the adenosine receptor and the prostaglandine receptor, was similar in both groups.

The analysis of lipolysis from individual concentration response curves is shown in Table 2. There was no difference in the basal lipolysis rate as well as in the lipolytic sensitivity of any of the agonists used (negative logarithm of EC_{50}) in the omental fat between the two groups. The responsiveness (lipolytic rate at maximum effective concentration) of norepinephrine, of the selective and nonselective β-adrenoceptor acting agents, and of all post adrenoceptor acting agents was increased by ~50% in PCOS, which was statistically significant for all agents except for dcAMP ($P = 0.056$). However, responsiveness for all antilipolytic agents was similar in the two groups. Only one drug concentration was used (1 mmol/l) for 8bcAMP. There was no significant correlation between insulin sensitivity (K_{itt} ranged between 3.2 and 6.5 for all subjects) and lipolytic responsiveness for any agent ($r < 0.31$).

There was a significant correlation between fat cell size and lipolytic responsiveness. The strongest correlation was observed for norepinephrine ($r = 0.64$, $P < 0.001$). Because there was a 25% (not significant) difference in fat cell size between the groups, we also corrected for this measure when values for lipolytic responsiveness in control and PCOS women were compared using ANCOVA. The findings were essentially the same as in Table 2. Thus, responsiveness for isoprenaline, dobutamine, terbutaline, CGP 12177, forskolin, dcAMP, and norepinephrine were increased in PCOS (*P* from 0.005 to 0.04).

Because there was a small difference in age between groups, the results in Table 2 were also compared by ANCOVA, with age as covariant. No significant effect of age on lipolysis data were seen. To further investigate the influence of age on lipolysis, data of both groups were put together, and a linear regression analysis was performed with age as regressor. There was no significant influence of age on lipolysis for any of the presented lipolysis data.

We also evaluated the influence of age by a retrospective analysis of previously published data on lipolysis in visceral fat cells (28). From this study, we selected data for basal lipolysis and isoprenaline responsiveness in all nonobese, premenopausal women (aged 26–49 years, BMI 19–26 kg/m^2, $n = 34$). Isoprenaline was used because it maximally activates lipolysis. Using linear regression analysis, no significant influence of age on basal ($r = 0.29$) or maximum isoprenaline ($r = 0.08$)-induced lipolysis was observed.

Protein isolation and Western blot analysis. Our results indicate that the increased lipolytic activity in PCOS subjects is due to an effect at the PKA-HSL level. This prompted us to examine possible alterations in the lipolytic signaling cascade by studying the protein expression of different components in the PKA complex, as well as HSL. The PKA complex is present as a heterotetramer composed of two catalytic and two regulatory subunits. The latter are subdivided into two classes (I and II), which in turn are present in two distinct isoforms (α and β). Previous studies have shown that the two major regulatory subunits expressed in fat cell are the RIα and RIIβ isoforms (28), and changes in the relative subunit expres-

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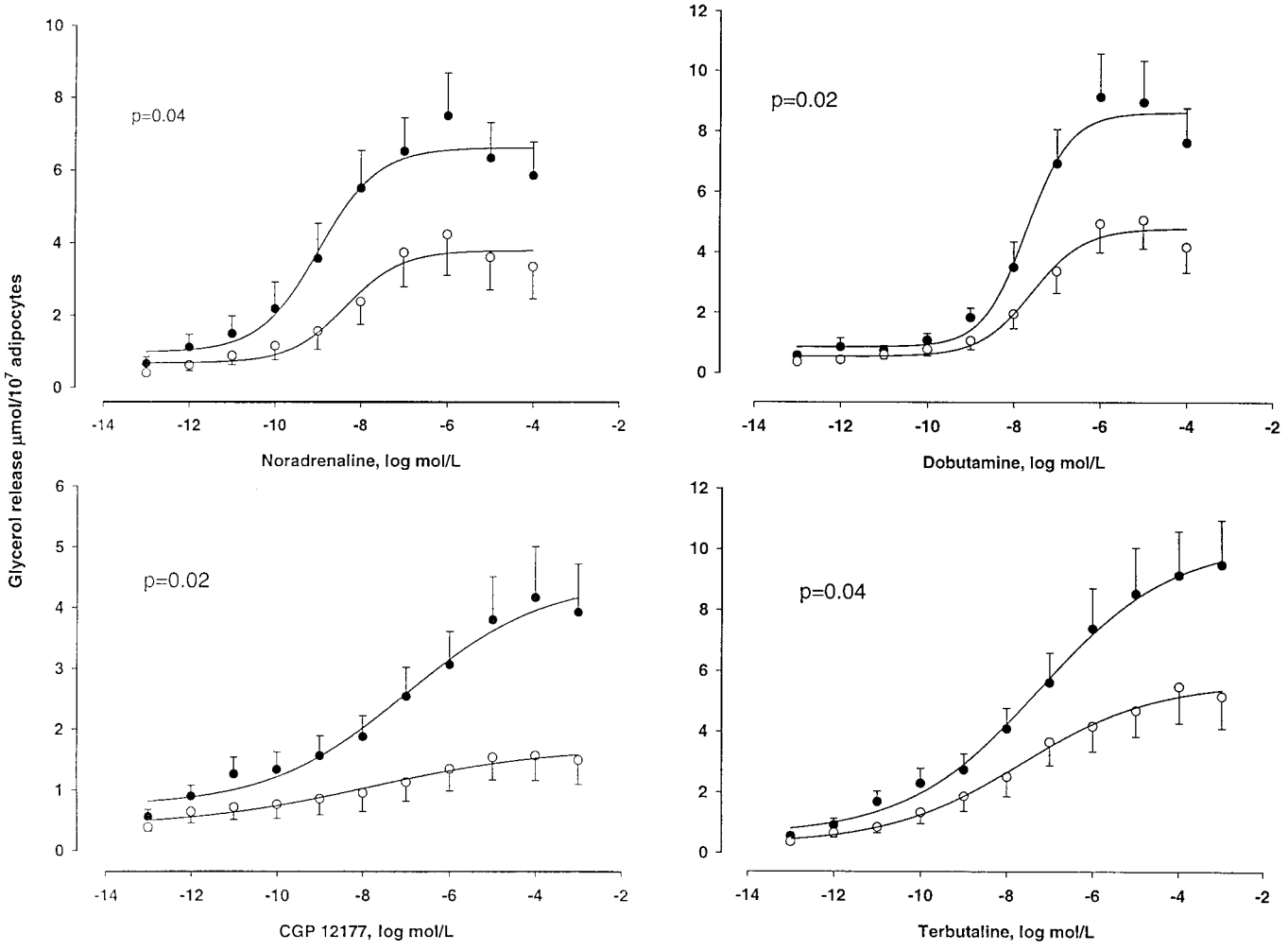

FIG. 1. Mean concentration response curves for the lipolytic effect (glycerol release) of adrenergic agonists in PCOS (●) and control women (○). Values are means ± SE. They were compared by ANOVA (two-way repeated measurements). *P* values are shown for the statistically significant comparisons.

sion can lead to altered intracellular signaling in animal models (29,30,31). HSL is the predominant lipase expressed in adipose tissue (32). In humans, a specific splicing of exon 6 occurs, resulting in the expression of two forms of HSL. One is a full-length protein with complete biological activity (HSL-long) and the other one is a truncated variant that is devoid of all lipase and esterase activity (HSL-short) (25).

Total protein lysates were obtained from omental adipose tissue samples of nine control and nine PCOS subjects and subjected to Western blot analysis (Fig. 4 shows data with PKA and Fig. 5 with HSL). Commercially available antibodies directed against the catalytic subunit of PKA (PKA cat), and the two regulatory subunits Iα (PKA RIα) and IIβ (PKA RIIβ) were acquired. A class I–specific antibody (PKA RI), directed against both class Iα and β isoforms, was also included in the studies. One of the co-authors (C.H.) provided two human-specific HSL antibodies, an exon 6–specific antibody as well as an antisera directed against both forms of human HSL (pan-HSL ab). Densitometric scanning and quantification of the blots showed that the expression levels of HSL-long were similar in all patients using both antibodies. Using the pan-HSL antibody, HSL-short was detected at lower but quantifiable levels in the control samples (204 ± 134 vs. 1,858 ± 389 OD · mm^{-2} · 100 μg total $protein^{-1}$ for HSL-long, $P < 0.01$). In contrast, expression of HSL-short was significantly lower in PCOS as compared with control samples ($P < 0.001$). In fact, HSL-short was barely detectable except in three PCOS samples. Analysis of the PKA complex expression showed that the protein levels of both the catalytic and one of the regulatory subunits (PKA RIα) were almost doubled in the PCOS subjects (PKA cat, 302 ± 19 and 496 ± 17 OD · 100 μg $protein^{-1}$ · mm^{-2}; PKA RIα, 54 ± 9 and 98 ± 11 OD · 100 μg $protein^{-1}$ · mm^{-2}, control and PCOS subjects, respectively, $P < 0.05$). Interestingly, expression of PKA RIIβ was reduced in PCOS as compared with control samples (951 ± 141 and 591 ± 47 OD · 100 μg $protein^{-1}$ · mm^{-2}, control and PCOS subjects, respectively, $P < 0.05$). Moreover, these alterations appear to be specific because probing with an antibody directed against both class I isoforms showed a similar expression of PKA RI in the two groups (81 ± 3 and 68 ± 5 OD · 100 μg $protein^{-1}$ · mm^{-2} in control and PCOS subjects, respectively, see Fig. 4*A*). This would imply that in PCOS subjects, a larger proportion of the PKA RI subunit is present in the α form.

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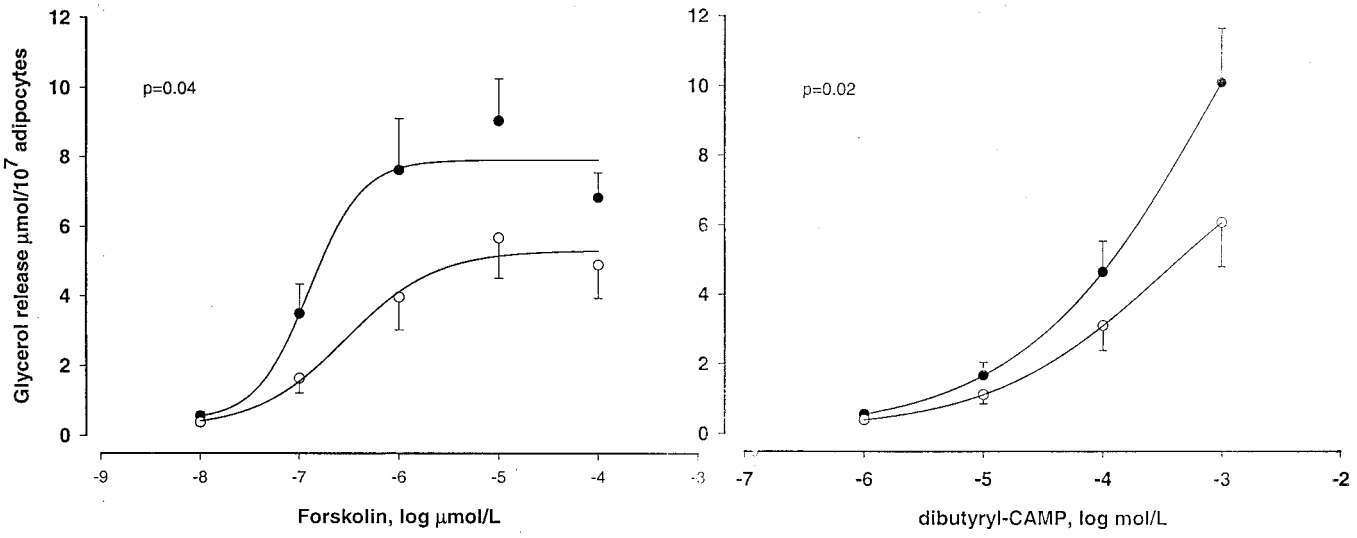

FIG. 2. Mean concentration response curves for the lipolytic effect of agents acting at postadrenoceptor levels. ●, PCOS women; ○, control women.

DISCUSSION

In this study, we investigated visceral adipose tissue lipolysis in PCOS. This is of relevance for type 2 diabetes, firstly, because of the putative role of visceral fatty acids for many abnormalities seen in the so-called insulin resistance (metabolic) syndrome and, secondly, because of the strong similarity between PCOS and the metabolic syndrome. To separate primary and secondary alterations in

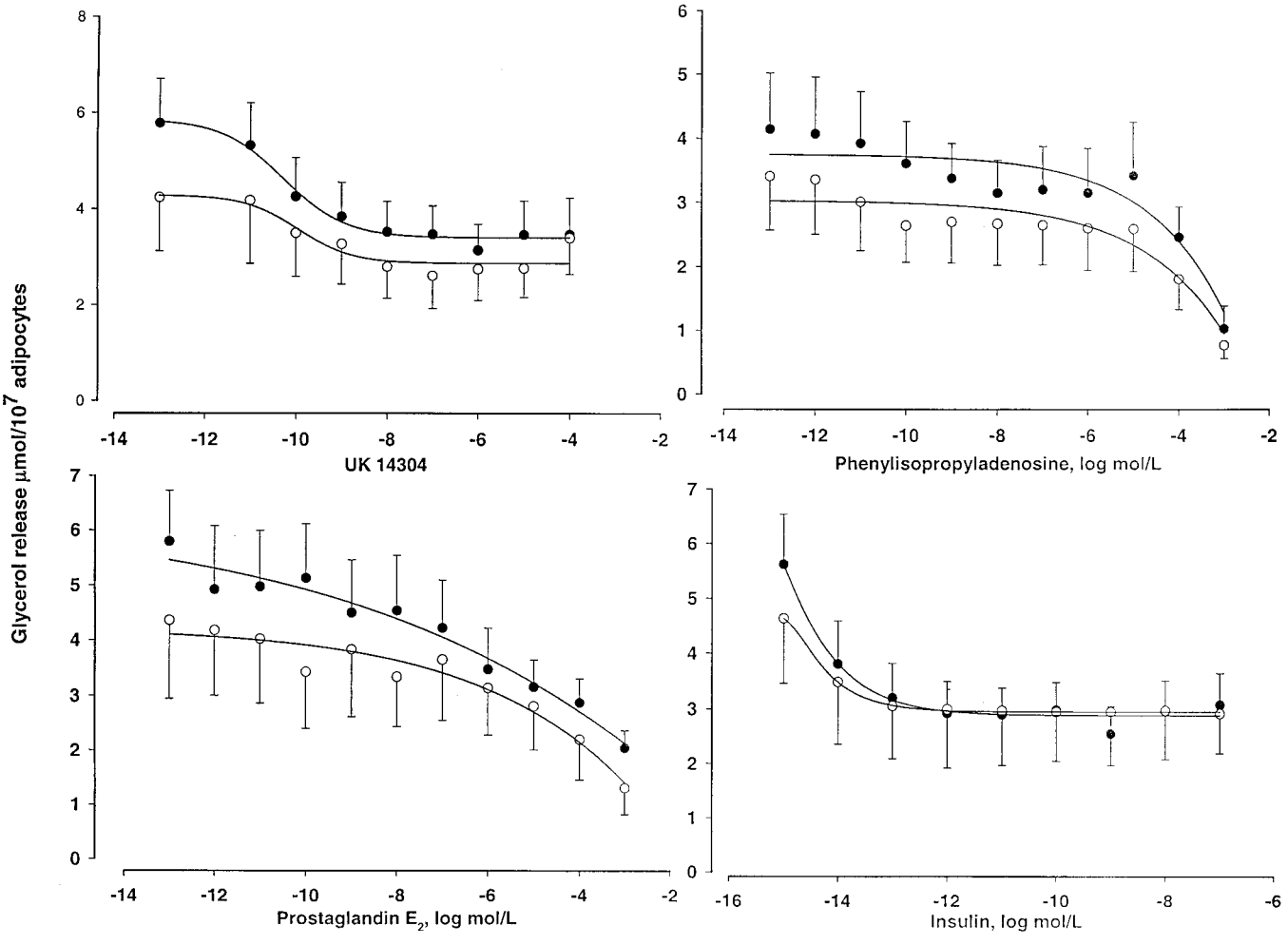

FIG. 3. Mean concentration response curves for antilipolytic agents. ●, PCOS women; ○, control women.

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TABLE 2
Lipolysis data

	Responsiveness		Sensitivity	
	PCOS	Control	PCOS	Control
Basal rate	0.6 ± 0.1	0.4 ± 0.1*	—	—
Isoprenaline	10.3 ± 1.3	6.1 ± 1.0†	9.37 ± 0.29	9.18 ± 0.23*
Dobutamine	9.4 ± 1.4	5.4 ± 1.0†	7.54 ± 0.16	7.58 ± 0.31*
Terbutaline	9.8 ± 1.5	5.8 ± 1.2‡	7.54 ± 0.16	7.58 ± 0.31*
CGP 12177	4.2 ± 0.4	1.7 ± 0.4‡	7.57 ± 0.49	8.42 ± 0.62*
Forskolin	9.5 ± 1.2	5.9 ± 1.2†	6.37 ± 0.16	6.11 ± 0.16*
dcAMP	10.1 ± 1.6	6.1 ± 1.3§	3.92 ± 0.10	4.93 ± 0.57*
Noradrenaline	7.7 ± 1.1	4.6 ± 1.1†	8.70 ± 0.28	8.40 ± 0.48*
8bcAMP	5.8 ± 0.9	4.2 ± 1.1	—	—
UK 14304	2.8 ± 0.5	2.4 ± 0.6*	9.41 ± 0.72	9.51 ± 0.29*
Phenylisopropyl adenosine	1.0 ± 0.4	1.1 ± 0.4*	9.47 ± 0.61	8.14 ± 1.29*
Prostaglandin E_2	2.0 ± 0.3	1.7 ± 0.6*	7.32 ± 0.87	7.18 ± 0.1*
Insulin	2.4 ± 0.5	2.4 ± 0.7*	13.46 ± 0.51	14.16 ± 0.27*

All values are means ± SE. Responsiveness: glycerol release (μmol · 10^7 cells^{-1} · 2h^{-1}) of maximum effective agonist concentration. Sensitivity is negative logarithm of half-maximum effective agonist concentration. Groups were compared using Student's unpaired two-tailed t test. *Not significant; †$P < 0.05$; ‡$P < 0.01$; §$P = 0.056$.

lipolysis, we investigated nonobese, young, and healthy PCOS women. These women showed no clinical abnormalities in body fat composition, lipid profile, or circulating insulin but already had a slight overall in vivo insulin resistance, as judged by the intravenous insulin tolerance. We admit that this is a crude measure of insulin sensitivity in vivo, but it does show a strong correlation with the more elaborate euglycemic-hyperinsulinemic clamp method (18). Probably due to the selection of the two study groups, PCOS women were slightly younger (mean 4 years) than control women. However, this minor age span has no important bearing on the results, as judged from present investigations on the influence of age on lipolysis.

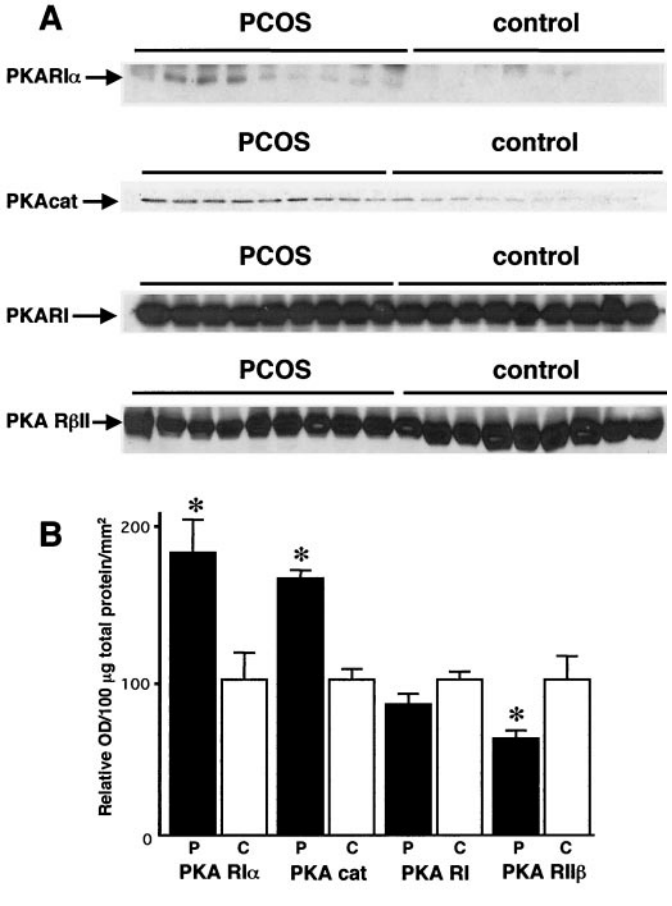

FIG. 4. Adipocyte protein content of components of the PKA complex. *A*: Western blots. *B*: Results of densitometry, compared by Student's unpaired *t* test. C, control women; P, PCOS women. *$P < 0.05$.

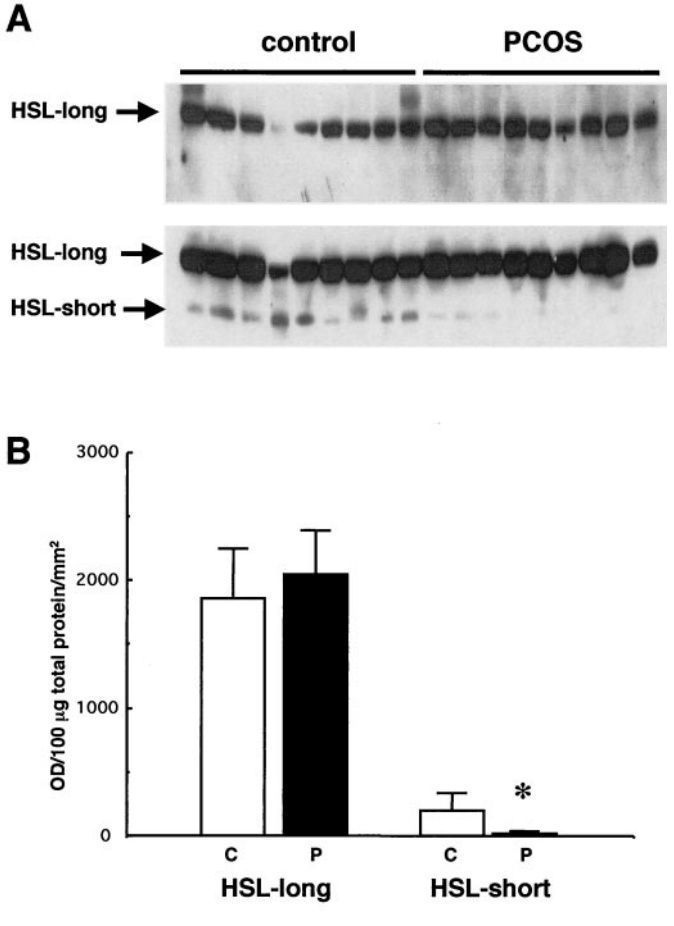

FIG. 5. Adipocyte protein content of the short and long forms of HSL. *A*: Western blots. *B*: Results of densitometry, compared by Student's unpaired *t* test. C, control women; P, PCOS women. *$P < 0.05$.

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Furthermore, recent data suggest a similar regulation of lipolysis in younger and elderly women (33).

There was a striking difference in the ability of norepinephrine to stimulate lipolysis in the visceral fat cells between the two groups. At all hormone concentrations (physiological and higher), the catecholamine effect was ~50% increased in PCOS women. Likewise, selective stimulation of lipolysis at selective β-adrenoceptor subtypes (with terbutaline, dobutamine, or CGP 17177), at all subtypes together (with isoprenaline), at the levels of adenylyl cyclase (with forskolin), or at PKA (with dcAMP) was also ~50% increased in PCOS women. These differences remained when statistical analysis was adjusted for a minor (nonsignificant) difference between groups in fat cell size. However, the antilipolytic properties of fat cells were normal in PCOS as judged by findings with insulin, adenosine, and prostaglandin analogs, as well as by selective activation of antilipolytic α-2 adrenergic receptors. These data strongly suggest a unique lipolysis upregulating alteration in visceral fat cells in PCOS due to a selective increase in the function of the PKA-HSL complex. This is in clear contrast to findings in insulin resistance (metabolic) syndrome, where catecholamine-induced lipolysis was increased in visceral fat cells due to a combination of increased β_3-adrenoceptor function and decreased α_2-adrenoceptor function (11,12).

Detailed molecular investigations of PCOS are limited by the fact that no relevant animal models are at hand (16). However, a mechanistic insight can be obtained from the analysis of protein levels of the various components of the PKA-HSL complex. Of the different catalytic and regulatory components of PKA, RIIβ is the most abundant in fat cells and seems to be the major determinant of fat cell lipolytic capacity (30,31). Studies in null mutant mice lacking the RIIβ gene have shown an increased rate of catecholamine-stimulated lipolysis in fat cells (31). This is probably due to a compensatory increase in the expression of the RIα isoform. The latter PKA regulatory component has a much stronger affinity for cyclic AMP than RIIβ and can therefore result in a more efficient and prolonged activation of the PKA-HSL complex. This leads to an enhanced breakdown of adipocyte triglycerides (31). In other mutant mice, it has also been demonstrated that the adipocyte level of the catalytic subunit determines the lipolytic capacity of fat cells (30). The results of the present investigation of the protein level profiles of PKA holoenzymes are in agreement with those from genetically modified mice models. Thus, we found decreased levels of RIIβ and increased levels of RIα and the catalytic component. As judged from animal data (30,31), this is predicted to cause an increment in catecholamine-stimulated lipolysis.

Another determinant of lipolysis capacity of human fat cells is the HSL level (26). Unlike HSL in rat fat cells, human HSL has two isoforms due to an alternative splicing of exon 6; the short form displays no enzymatic activity (25). HSL-short lacks the catalytic serine residue but retains the phosphorylation sites (present in exon 8) and can thus function as a substrate for PKA. Protein levels of HSL-long were not altered in PCOS women. In contrast, PCOS fat cells were almost completely devoid of HSL-short, which was present at ~10% of the level of the long form in control tissue. The pathophysiological meaning of this finding is unclear at the moment. However, it is conceivable that expression of an enzymatically inactive HSL that can still interact with PKA may function in a dominant-negative mode. Thus, a low or absent expression of HSL-short may contribute to a more efficient activation of lipolysis. In animal models, adipocyte HSL levels are not altered in mice with null mutations for different components of PKA (30,31). We admit that the results of HSL expression are of observational character and need to be validated in further in vitro experiments. Most importantly, however, such studies can only be performed on human tissue or cells because the HSL splicing is specific for our species. Unfortunately, the amount of adipose tissue that can be obtained under the present laparoscopic procedures is too small for more detailed investigations.

It appears from studies on HSL that lipolysis in fat cells can be activated by alternative and not yet defined lipases (32). These lipases might also be involved in the increased lipolytic responsiveness of visceral fat cells in PCOS.

At the moment, we have no clear answer as to whether lipolytic activity of visceral fat in vitro is causative for the in vivo insulin resistance or whether the two observations are correlative. There was no significant direct correlation between lipolysis and insulin sensitivity. This might have resulted from the range of K_{itt} values being narrow and because K_{itt} is an overall measure. Perhaps a more specific analysis of hepatic or peripheral insulin sensitivity might have revealed a correlation. Although hyperandrogenic women have more peripheral than hepatic insulin resistance (34), it is unknown how this relates to true PCOS. The insulin resistance in PCOS appears to be unique for this condition, involving insulin-independent phosphorylation of serine in the β-subunit of the insulin receptor (35). The present observation of a normal antilipolytic effect of insulin in visceral fat cells in PCOS supports the idea that the measured in vivo insulin resistance of the glucose-lowering effect of the hormone is secondary, perhaps due to elevated "portal" free fatty acids. Such fatty acids could interfere with insulin signaling or interact with the liver so that the hypothalamus-pituitary-adrenal axis and sympathetic activation are disturbed, leading to insulin resistance, as discussed (36,37). Fatty acids might also reduce the liver production of SHBG due to gene expression effects (38) or by inducing some liver inflammation (39) so that a hyperandrogenic state occurs.

There is some recent evidence for a role of visceral adipose tissue for liver function. Selective removal of visceral fat improves liver function in rodents (40). Removal of the major omentum in connection with bariatric surgery for obesity improves insulin sensitivity, hyperinsulinemia, and glucose intolerance independently of a decrease in body weight (41).

A prominent feature of PCOS is hyperandrogenemia, which was also found in the present study. Studies on rat adipocytes suggest that testosterone improves catecholamine-stimulated lipolysis due to multiple alterations in signal transduction involving PKA-HSL (42,43). However, it is not probable that the increased lipolysis alteration in PCOS is a secondary phenomenon due to high androgen levels. Oral contraceptive treatment, which normalizes androgen levels, does not improve catecholamine-induced lipolysis in subcutaneous adipose tissue of lean (14) or

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obese PCOS women (15). A selective upregulation of lipolysis in visceral fat by androgens seems less likely because testosterone receptors are present in subcutaneous and visceral fat depots, although they are more abundant in the visceral region (44).

Although care should be exercised when extrapolating in vitro data to the situation in vivo, on the basis of the present and previous (14) data on lean young and apparently healthy PCOS women, we propose the following original hypothesis for the diabetic phenotype of PCOS. There are early and possibly primary defects in catecholamine-induced lipolysis. The lipolytic effect is decreased in subcutaneous fat cells due to decreased β_2-adrenoceptor expression and decreased PKA-HSL function, which is similar to the defect in insulin resistance (metabolic) syndrome (13). In visceral fat cells, the lipolytic effect is increased as a result of unique alterations of the stoichiometric properties of the PKA-HSL complex that are different from the defects seen in insulin resistance (metabolic) syndrome, which are localized at the adrenoceptor level (12). The combination of subcutaneous resistance and increased visceral action will redistribute the catecholamine effect on lipolysis, favoring the release of fatty acids from the visceral fat depot. This, in turn, increases the delivery of fatty acids and glycerol to the liver, thereby inducing insulin resistance in otherwise apparently healthy young PCOS subjects and starting a vicious circle. The development of obesity and/or upper body fat distribution later in life might further increase visceral fat cell lipolysis, as seen in insulin resistance (metabolic) syndrome, so that dyslipidemia, hyperinsulinemia, and glucose intolerance develop. We admit that this idea is based on a cross-sectional in vitro study. Unfortunately, it is not possible, for ethical reasons, to study visceral fat cells longitudinally or to directly perform investigations of visceral fat lipolysis in vivo, because the latter requires access to the portal vein.

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