Potential effects of aerobic exercise on the expression of perilipin 3 in the adipose tissue of women with polycystic ovary syndrome: a pilot study

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Abstract

Objective: Polycystic ovary syndrome (PCOS) is associated with reduced adipose tissue lipolysis that can be rescued by aerobic exercise. We aimed to identify differences in the gene expression of perilipins and associated targets in adipose tissue in women with PCOS before and after exercise.

Design and methods: We conducted a cross-sectional study in eight women with PCOS and eight women matched for BMI and age with normal cycles. Women with PCOS also completed a 16-week prospective aerobic exercise-training study. Abdominal subcutaneous adipose tissue biopsies were collected, and primary adipose-derived stromal/stem cell cultures were established from women with PCOS before 16 weeks of aerobic exercise training (n = 5) and controls (n = 5). Gene expression was measured using real-time PCR, in vitro lipolysis was measured using radiolabeled oleate, and perilipin 3 (PLIN3) protein content was measured by western blotting analysis.

Results: The expression of PLIN1, PLIN3, and PLIN5, along with coatomers ARF1, ARFRP1, and βCOP was ~80% lower in women with PCOS (all P < 0.05). Following exercise training, PLIN3 was the only perilipin to increase significantly (P < 0.05), along with coatomers ARF1, ARFRP1, βCOP, and SEC23A (all P < 0.05). Furthermore, PLIN3 protein expression was undetectable in the cell cultures from women with PCOS vs controls. Following exercise training, in vitro adipose oleate oxidation, glycerol secretion, and PLIN3 protein expression were increased, along with reductions in triglyceride content and absence of large lipid droplet morphology.

Conclusions: These findings suggest that PLIN3 and coatomer GTPases are important regulators of lipolysis and triglyceride storage in the adipose tissue of women with PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine and reproductive disorder affecting ~4–7% of women of reproductive age (1, 2). As a principle cause of infertility in reproductive aged women, PCOS is characterized by the presence of menstrual disturbances, hyperandrogenemia, and ovarian cysts (3). Similarly, ~70% of women with PCOS have increased adiposity (4), and between 20 and 43% have insulin resistance and reduced glucose control (5, 6). One possible culprit speculated to contribute to this irregular metabolic phenomena is defects within the adipose tissue (7).

Adipose tissue functions as a storage reservoir for excess lipid, a reserve that must be readily lipolyzed upon...
increased energy demand. Inefficient ability of the adipose tissue to mobilize and secrete free fatty acids during conditions of energy demand has been linked with the conditions of impaired glucose tolerance and type 2 diabetes (8, 9, 10). It has been previously documented that women with PCOS have dysfunctions in lipase activity and catecholamine-mediated lipolysis (11, 12, 13). Furthermore it has been reported that testosterone can reduce: i) catecholamine-stimulated adipose tissue lipolysis (14, 15); ii) expression of β-adrenergic receptors in adipose tissue (14, 15); and iii) adrenergic stimulated lipolysis in the brown adipose tissue (16). Possible molecular targets, such as the expression of lipases, have been implicated as factors regulated by testosterone (15, 17, 18), thus offering a possible relationship between impaired lipolysis and the hyperadrogenemic states seen in women with PCOS.

Exercise training has been shown to improve several metabolic impairments typical of PCOS (19, 20, 21), and has been shown to enhance adipose tissue lipolysis (22, 23). Previous studies from our group revealed an increase in basal and pharmacologically stimulated lipolysis with isoproterenol from the adipose tissue following exercise training (24). The molecular markers that regulate lipolysis in the adipose tissue of women with PCOS however have not been thoroughly studied.

Prior investigations have shown that a single-nucleotide polymorphism within the perilipin gene (PLIN1) that exists in women with PCOS is associated with impaired glucose tolerance and increased LDL (25), perhaps implicating a potential role for the perilipin family of proteins, lipases, and related factors affecting adipose tissue lipolysis. Recent investigations have also identified several coatomer GTPase proteins (ADP-ribosylation factor 1 (ARF1), SEC23A, coatomer complex 1 subunit beta (β-COP1), GBF1, and ARF-related peptide 1 (ARFRP1)), which are usually involved in endoplasmic reticulum (ER)-to-Golgi transport, and which are involved in the delivery of adipose tissue triglyceride lipase (ATGL) to lipid droplets, particularly those lipid droplets coated by perilipin 3 (PLIN3) (26). We have recently shown that PLIN3 and coatomer GTPases in skeletal muscle tissue and in primary human skeletal muscle cultures were associated with increased fat oxidation following exercise and lipolytic stimulation (27). We, therefore, proposed a hypothesis that these novel mediators of lipolysis might also be differentially expressed in the adipose tissue of women with PCOS, where lipolysis is impaired and upregulated following aerobic exercise training. We investigated the expression of lipases, perilipins, and coatomer GTPases from adipose tissue in a cross-sectional cohort of eight women with PCOS compared with women with normal menses matched for age, BMI, and percent body fat. Furthermore, in the women with PCOS, we investigated the effects of a 16-week aerobic exercise training program on lipolysis both in adipose tissue and in stromal-derived adipose cultures. Our results indicate previously the unexplored potential roles for PLIN3 and coatomers (ARF1, ARFRP1, β-COP1, and coatomer complex 2 subunit 23a (Sec23a)) in the regulation of lipolysis in the adipose tissue.

**Materials and methods**

**Participants and study design**

Eight obese women with PCOS and eight age-, BMI-, and percent fat-matched healthy women without clinical signs of abnormal menses or hyperandrogenemia were recruited in this study (anthropometric characteristics are provided in Table 1). The results reported for this investigation were an ancillary project to a clinical study, which was designed and powered to determine the effect of an aerobic exercise program on body composition and whole-body insulin resistance in obese women diagnosed with PCOS. The original study was powered using the mean and s.d. of glucose disposal rate (GDR) to estimate sample sizes required to measure a minimum 20% change in GDR from baseline. With a prospective study design (paired) with a target power of 80% and a significance level set to α = 0.05, we can conclude that only six subjects would be needed to detect a 20% change in GDR from baseline. The main outcomes of this study were previously reported in Moro et al. (24) and in Redman et al. (28). The diagnosis of PCOS was assessed by the Rotterdam criteria (3, 29).

Women with PCOS had to possess two of the following criteria: confirmation by medical history of menstrual irregularity (oligo- or amenorrhea), presence of more than ten ovarian follicles of 2–9 mm in diameter as assessed by magnetic resonance imaging (MRI) or either clinical (hirsutism score) or serum measures of androgen excess (elevated free androgen index (FAI)). Other causes of oligomenorrhea (hyperprolactinemia, congenital adrenal hyperplasia, Cushing’s syndrome, and hyperthyroidism) were excluded by medical history. Women in the control group were excluded from participating in our study for exercise training, use of contraceptive medications, and menstrual cycle irregularity together with androgen excess. All women in our control group had FAI values below 3.6 as defined as a cut-off value for FAI in the assessment of PCOS according to Hahn et al. (30).
Table 1  Anthropometric, metabolic, and serum markers for both the cross-sectional study between controls and women with PCOS, and the exercise-training intervention in the women with PCOS only. This data reflect the five females with PCOS for whom we were able to obtain primary stromal-derived adipose cultures both before and after exercise intervention.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Women with PCOS</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>Pre-exercise</td>
<td>Post-exercise</td>
<td>Control vs PCOS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre- vs post-exercise</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>29.7±12.4</td>
<td>27.0±2.9</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>78.9±15.7</td>
<td>82.2±18.2</td>
<td>0.74</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>26.5±5.4</td>
<td>30.4±4.2</td>
<td>0.17</td>
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<tr>
<td><strong>Total body fat (%)</strong></td>
<td>29.9±9.8</td>
<td>36.8±5.0</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>FM (kg)</strong></td>
<td>23.3±9.3</td>
<td>30.8±11.1</td>
<td>0.23</td>
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<tr>
<td><strong>FFM (kg)</strong></td>
<td>55.6±15.9</td>
<td>51.4±7.9</td>
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<td><strong>Visceral AT (kg)</strong></td>
<td>1.4±0.8</td>
<td>1.2±0.5</td>
<td>0.75</td>
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<td><strong>Subcutaneous AT (kg)</strong></td>
<td>11.4±1.8</td>
<td>10.9±4.8</td>
<td>0.86</td>
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<tr>
<td><strong>Insulin (mg/dl)</strong></td>
<td>0.013±0.017</td>
<td>0.13±0.17</td>
<td>0.16</td>
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<tr>
<td><strong>IMCL (soleus, AU)</strong></td>
<td>0.006±0.003</td>
<td>0.006±0.004</td>
<td>0.89</td>
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<td><strong>Glucose, fasting (mg/dl)</strong></td>
<td>103.5±28.6</td>
<td>82.2±6.3</td>
<td>0.14</td>
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<tr>
<td><strong>Insulin, fasting (mg/dl)</strong></td>
<td>11.0±10.2</td>
<td>9.8±3.9</td>
<td>0.81</td>
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<tr>
<td><strong>HOMA-IR (AU)</strong></td>
<td>3.6±4.2</td>
<td>2.0±0.8</td>
<td>0.50</td>
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<td><strong>GDR/EMBS (mg/kg FM ±17.7)</strong></td>
<td>6.8±4.3</td>
<td>6.3±1.2</td>
<td>0.79</td>
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<tr>
<td><strong>Resting metabolic rate/FFM (kcal/day per kg)</strong></td>
<td>30.3±4.8</td>
<td>30.7±5.0</td>
<td>0.91</td>
</tr>
<tr>
<td><strong>Resting RQ</strong></td>
<td>0.80±0.03</td>
<td>0.81±0.02</td>
<td>0.37</td>
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<td><strong>Clamp RQ</strong></td>
<td>0.93±0.07</td>
<td>0.90±0.04</td>
<td>0.50</td>
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<tr>
<td><strong>ΔRQ (clamp vs resting)</strong></td>
<td>0.13±0.07</td>
<td>0.09±0.02</td>
<td>0.26</td>
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<td><strong>VO₂max (ml/min per kg)</strong></td>
<td>27.7±10.0</td>
<td>29.5±3.0</td>
<td>0.71</td>
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<td><strong>Fat cell size (nl)</strong></td>
<td>0.69±0.31</td>
<td>0.68±0.13</td>
<td>0.92</td>
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<td><strong>DHEA-S (µg/ml)</strong></td>
<td>135.3±49.6</td>
<td>159.0±73.6</td>
<td>0.47</td>
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<tr>
<td><strong>Testosterone (ng/ml)</strong></td>
<td>35.4±11.7</td>
<td>92.4±41.2</td>
<td>0.002</td>
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<tr>
<td><strong>SHBG (nmol/l)</strong></td>
<td>24.1±4.6</td>
<td>30.9±31.7</td>
<td>0.56</td>
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<tr>
<td><strong>FAI (AU)</strong></td>
<td>1.2±0.6</td>
<td>18.8±14.2</td>
<td>0.02</td>
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<tr>
<td><strong>Number of ovarian cysts</strong></td>
<td>29.4±46.1</td>
<td>97±29.7</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>(2–9 mm in diameter)*</td>
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<td>0.10</td>
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EMBS, estimated mean body size; FM, fat mass; FFM, fat free mass; GDR, glucose disposal rate; HOMA-IR, Homeostatic model assessment of insulin resistance; RQ, respiratory quotient; SHBG, sex hormone-binding globulin; FAI, free androgen index; AT, adipose tissue; IHL, intrahepatic lipid; IMCL, intramyocellular lipid.

Follicles measuring between 2-9mm in diameter were only detectable in 3 of the 8 control participants, but all women with PCOS possessed greater than 10 follicles measuring between 2-9mm in diameter.

In addition, all women in our control group reported regular menstruation, thus confirming that they did not have PCOS based on the Rotterdam guidelines. The potential subjects were excluded from participating in either group if they: smoked; were taking any medications; had current or past history of cardiovascular disease; hypertension (>140/90 mmHg); diabetes (type 1 or type 2); kidney, liver, or heart disease; alcoholism or substance abuse; were pregnant or trying to become pregnant; and were unable to comply with the exercise training program. Adipose tissue measures are reported in all participants included in this study. Fat cell size was determined using osmium fixation and counted using a multisizer 3 Coulter counter (Beckman Coulter, Brea, CA, USA) as previously described (31). In vitro measures of stromal-derived adipocytes are reported in five women with PCOS before and after exercise due to the availability of adipose tissue material for the isolation and culturing of stromal-vascular cells. For immunocytochemistry analysis of lipid droplet comparisons and protein immunoblotting, controls were taken from five women with normal menstrual cycling unrelated to the original study that were matched for age (24.2±2.3 years, P=0.37 compared to women with PCOS) and BMI (26.1±2.5 kg/m², P=0.83 compared with women with PCOS) – this was necessary because the collection of stromal-derived adipose cultures was not part of the initial study protocol for the control subjects. The study design and protocol were approved by the Institutional Review Board of Pennington Biomedical Research Center, and all volunteers gave written informed consent. This study was registered at clinicaltrials.gov (NCT01150539).

All women were examined at baseline, and the women with PCOS were reexamined after 16 weeks of aerobic exercise training. At each time-point, study testing occurred over 3 days. In order to reduce confounding...
factors associated with dietary differences and metabolic testing, we provided participants with a standard diet of 50% carbohydrate, 35% fat, and 15% protein 2 days before testing and throughout the duration of testing. Following an overnight fast, blood and subcutaneous abdominal adipose tissue samples were collected, body composition (percent body fat surmised from all regions of the body) was assessed by dual-energy X-ray absorptiometry (DXA, QDR 4500A; Hologic, Bedford, MA, USA), and insulin sensitivity was determined by a hyperinsulinemic–euglycemic clamp (120-min at 80 mU/min per m²) as previously described (28). Aerobic capacity (VO_{2max}) was measured during a graded treadmill test (TrueMax 2400; ParvoMedics, Salt Lake City, UT, USA). Serum testosterone and sex hormone-binding globulin (SHBG) were determined by an automated chemiluminescent immunoassay on the Immulite 2000 (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Serum glucose and serum insulin were measured by an enzymatic assay on a Beckman Coulter DXC 600 (Beckman Coulter). Ovarian morphology, abdominal subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT) as well as intrahepatic lipid and intramyocellular lipid (IMCL) of the soleus were examined before and after 16 weeks of aerobic exercise training using a 3T MRI/MRS (GE 3.0T Signa EXCITE MRI, GE Healthcare, Pittsburgh, PA, USA; results provided in Table 1).

**MRI and spectroscopy**

Abdominal fat: an 8-channel torso-array coil was placed over the chest/abdomen area and 3.4 mm slices (1.7 mm intersection gap) were acquired from the highest point of the liver to the inferior pole of the right kidney. A total of ~220 images were acquired on each participant. Total adipose tissue, VAT, and subcutaneous abdominal adipose tissue masses were calculated using the Analyze Software Package (CNSoftware, Rochester, MN, USA). Muscle and hepatic lipid content: for the muscle IMCL measures, the right leg was positioned inside a 1H knee coil with the knee in extension and the ankle in a neutral position. Separate water-suppressed PRESS boxes (10×7.5×7.5 mm voxels) were collected from the tibialis anterior (n=1), soleus (n=3), and peanut oil phantom (n=1). For the liver lipid content, with the participant lying prone, a 1H body coil was placed over the torso and a single PRESS box (30×30×30 mm) was collected in an area of the liver free from heavy vascularization. Data were analyzed using the jMRUI Software Package (Lyon, France). Ovarian morphology: an 8-channel torso array coil was used to acquire coronal (T2-weighted fast spin echo (FSE), short T1 inversion recovery (STIR), and T1-weighted localizer), sagittal (T1-weighted localizer only), and axial (T2-weighted FSE and STIR) sequences. Images were acquired from the highest point of the uterus or ovaries through the bottom of the ovaries with 4-mm thick slices and 1 mm intersection gap for a total of ~40 images. Images were analyzed using Analyze 8.1 (CNSoftware) by a trained analyst. The ovaries, as well as ovarian follicles, were located and quantified on each coronal FSE image. The follicles were located on an image and followed down through the remaining consecutive images to ensure that each individual follicle was identified as such. The total number of follicles in each ovary was calculated using this technique. The volume of each follicle was calculated based on the number of pixels measured in each image and the number of consecutive images in which the follicle was located.

**SAT biopsy**

SAT was obtained from the left upper quadrant of the abdomen with a 5-mm Bergstrom needle using the Bergstrom technique. The skin was cleansed with povidone-iodine solution, a sterile drape was placed over the incision site, and local anesthesia (5 ml 1:1 mixture of 0.5% bupivacaine and 2% lidocaine) was administered. An ~1 cm incision was made and adipose tissue was collected.

**Exercise training program**

Aerobic exercise was performed under supervision at the Pennington Health and Fitness Center, five times per week. Aerobic exercise was prescribed on an individual basis with the objective to achieve specified exercise energy expenditure (ExEE) in each session. During the first 4 weeks, the target ExEE was 4% of the participants’ estimated energy requirement for weight maintenance, and was incremented to 6% for weeks 5–8, to 8% for weeks 9–12, and to 10% for weeks 13–16. All exercises were performed on a treadmill at 55% VO_{2max}, a moderate intensity. The necessary speed and gradient to achieve the ExEE were estimated from a linear regression of O2 uptake and workload during the VO_{2max} test, and the ExEE was confirmed once during each 4-week interval by indirect calorimetry. Heart rate was monitored during all sessions to verify ExEE. The exercise time necessary to complete the energy expenditure target was 23±1 min/session during weeks 1–4, 35±1 min/session during weeks 5–8, 47±2 min/session during weeks 9–12, and 58±2 min/
session during weeks 13–16. In order to prevent variation in results due to dietary influences during the exercise intervention, we asked participants to maintain their dietary habits throughout the 16-week intervention as they had before study enrollment. In addition, we requested updates on food intake during site visits for exercise sessions.

Stromal-derived adipocyte cultures, immunofluorescence staining, lipolysis, triglyceride determination, and oleate oxidation

Adipose tissue samples from subcutaneous abdominal depot were collected under aseptic conditions and isolated stromovascular (SV) cells were cultured as previously described (32) and differentiated as described previously (33) with modifications: DMEM–F12 (1:1) medium plus 10 mg/ml transferrin, 33 μM biotin, 17 μM calcium pantothenate, 0.5 μM insulin, 0.1 μM dexamethasone, 0.2 nM triiodothyronine, as well as 0.5 μM rosiglitazone and 540 μM 3-isobutyl-1-methylxanthine (IBMX) during the last 48 h of culture. All experiments were performed on cells following 9 days of differentiation. The cultures were stained for lipids (BODIPY, 10 μM) and DNA (4,6-diamidino-2-phenylindole (DAPI), 300 nM) (Invitrogen). The images were obtained using the Leica TCS SP5 AOBS resonant scanning confocal microscope (Leica AG, Wetzlar, Germany). The lipolysis assay was performed over 3 h by adding 0.2 ml of HBSS + BSA 2%. At the end of incubation, the medium was collected for the measurement of glycerol, performed in duplicate using the free glycerol reagent (Sigma–Aldrich), and adjusted for the triglyceride content. Triglyceride levels were then measured using a glycerol phosphate oxidase triglyceride determination kit (Sigma–Aldrich) and normalized according to the protein content. The cultures were preincubated for 3 h with [1-14C] oleic acid (1 μCi/ml; PerkinElmer, Boston, MA, USA) and non-labeled (cold) oleic acid (100 μM). Oleic acid was coupled with fatty acid: free BSA in a molar ratio of 5:1. Following incubation, 14CO2 was measured as described previously (34). The cells were then lysed in 0.2 ml of 0.1% SDS for the determination of cell-associated label uptake and protein content for normalization. All assays were performed in duplicate.

Real-time qRT-PCR and western blotting

Total RNA was extracted from ~100 mg of adipose tissue using the miRNEasy Kits (Qiagen) according to the manufacturer’s specifications. RNA extracts were converted into cDNA using the High-Capacity cDNA RT Kit (Applied Biosystems) and stored at −20 °C until real-time PCR was performed. Gene expression was carried out using real-time PCR with TaqMan gene expression assays on demand on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Gene expression assays were performed for the following genes: PLIN1 (Hs00160173_m1), PLIN2 (Hs00765634_m1), PLIN3 (Hs00998421_m1), PLIN4 (Hs00287411_m1), PLIN5 (Hs00965990_m1), G6P1 (Hs00188327_m1), ARFI (Hs00796826_s1), ATGFRP1 (Hs00182389_m1), GAPDH (Hs00200752_m1), DGAT2 (Hs01045913_m1), mPGAT (Hs00326039_m1), and PPIA (Hs009999904_m1). Relative gene expression was assessed using a standard curve of known concentrations of mRNA and normalized for cyclophilin A (PPIA) gene expression. Protein extracts from adipose culture were immunoblotted and probed with an antibody against PLIN3 (Novus Biologicals, Littleton, CO, USA) and normalized against the loading control GAPDH (Abcam, Cambridge, MA, USA).

Statistical analyses

All analyses were performed using GraphPad Prism Software, version 5.0 (GraphPad Software, La Jolla, CA, USA). The Mann–Whitney U test was used for cross-sectional comparisons between PCOS and obese control women when data were not normally distributed, and an independent samples t-test was used when the data were normally distributed. The paired t-tests were used to compare variables from baseline with the end of the exercise training intervention, except when the data were not normally distributed, Wilcoxon signed-ranked paired tests were used. P<0.05 was considered statistically significant. All graphical data are presented as mean ± S.E.M.

Results

Comparison of controls vs women with PCOS

Compared with the control participants, women with PCOS had elevated serum testosterone and FAI, as expected, but they did not differ in terms of SHBG (Table 1). Otherwise, control participants were effectively matched with women with PCOS for body composition, insulin sensitivity (fasting glucose, fasting insulin, GDR, and HOMA-IR), and energy metabolism (resting metabolic rate, fasting respiratory quotient (RQ), metabolic
flexibility (i.e. changes between the fasting RQ and RQ during the steady state of the hyperinsulinemic-euglycemic clamp), and VO2\textsubscript{max} (Table 1). Importantly, women with PCOS did not differ from control participants with regard to mean subcutaneous fat cell size (Table 1).

Women with PCOS did however differ significantly from controls in terms of SAT gene expression for the candidates responsible for lipolysis, lipid droplet perilipin proteins, and coatomer proteins. As shown in Fig. 1A, PLIN1, PLIN3, and PLIN5 were \sim 80–90% lower in women with PCOS when compared with controls, while PLIN2 and PLIN4 were approximately threefold higher. Expression mRNA levels of coatomer GTPases ARF1, ARFRP1, and βCOP (Fig. 1B) were \sim 80% lower in women with PCOS compared with controls, with GBF1 and SEC23A being expressed approximately nine- and sevenfold higher respectively. Finally, lipase mRNA expression of ATGL and monoglycerol lipase (MGL) was reduced by 90 and 65%, respectively, with no difference in the expression of CGI58 (Fig. 1C).

**Effect of 16-week aerobic exercise training in women with PCOS**

Aerobic exercise resulted in a \sim 10% improvement in maximal aerobic capacity (VO2\textsubscript{max}, \textit{P}=0.04) and a \sim 20% improvement in GDR (\textit{P}=0.02) (Table 1). No other changes were noted in terms of body composition, abdominal adipose depot size (visceral or subcutaneous depots), ectopic lipid accumulation (in the liver or muscle), or fat cell size (Table 1). However, alterations were seen in the gene expression in the adipose tissue following exercise. Of all the measured perilipins, only expression of PLIN3 increased significantly (\textit{P}<0.05, Fig. 2A), whereas PLIN1, PLIN2, PLIN4, and PLIN5 did not change. On average, expression of ARF1, ARFRP1, βCOP, and SEC23A was increased by approximately five-, eight-, seven-, and fourfold, respectively (\textit{P}<0.05), with no changes in GBF1

**Figure 1**
Gene expression from abdominal subcutaneous adipose tissue from women with PCOS vs age, BMI, and percent fat matched controls (cross-sectional study). (A) Perilipin family of protein gene expression of all five perilipin proteins shows drastic differences in expression. Perilipin 1 (PLIN1), PLIN3, and PLIN5 are all expressed \sim 90% lower in females with PCOS compared to controls. PLIN2 and PLIN4 are expressed about three times higher when compared with controls. (B) Coatomer proteins involved in ER-to-Golgi transport were also differentially regulated on the gene expression level. ADP-ribosylation factor 1 (ARF1), ARF-related protein 1 (ARFRP1), and beta-coatomer (βCOP, part of the COPI complex) were drastically reduced in women with PCOS. GDP-exchange brefeldin A resistant factor 1 (GBF1, which operated in conjunction with ARF1) and Sec23a (part of the COPII complex) were both expressed approximately eightfold higher in the women with PCOS. (C) Expression of adipose triglyceride lipase (ATGL) and monoglyceride lipase (MGL) were both reduced in women with PCOS on the gene expression level, while there was no statistical difference in the expression of the ATGL co-activator CGI58. *\textit{P}<0.05; **\textit{P}<0.01; and ***\textit{P}<0.001.
In addition, the lipases ATGL, MGL, and the ATGL co-activator CGI58 increased significantly after 16-week exercise training ($P<0.05$, Fig. 2C).

Primary adipocyte culture lipid droplet morphology and lipolysis

As shown in Fig. 3, primary adipocyte cultures revealed a larger lipid droplet morphology in women with PCOS compared with controls, which decreased in size after exercise training, and thus more closely resembled the morphology of control females (Fig. 3). In vitro measures of fat oxidation and lipolysis in primary cultured adipocytes showed that oleate oxidation and glycerol release were increased following exercise training ($P<0.05$, Fig. 4A and B). Likewise, total triglyceride content was reduced after exercise ($P<0.05$, Fig. 4C).

Figure 2
Gene expression targets in adipose tissue known to be involved in lipid droplet regulation and lipolysis were altered at 16 weeks of exercise training in the women with PCOS (exercise training intervention, women with PCOS only). (A) Gene expression of the perilipin family of proteins from before to after exercise demonstrates that only PLIN3 is significantly elevated following exercise training. (B) Coatomer gene expression reveals that ARF1, ARFRP1, and βCOP, which were grossly decreased when compared with controls before exercise training, revealed an increase in their expression. Sec23a was also increased following exercise training. (C) Gene expression of ATGL and MGL, which was blunted when compared with controls at baseline, increased with exercise training. Additionally, CGI58 increased following exercise training. *$P<0.05$.

Figure 3
Representative images of stromal-derived pre-adipocytes cultured and differentiated into adipocytes. Women with PCOS possessed large lipid droplets when compared with control donors matched by age, BMI, and % fat. Lipid droplet morphology appeared to resemble that of controls in our cross-sectional study following 16 weeks of aerobic exercise training in women with PCOS. Bodipy 494 (green) was used to identify lipid and DAPI (blue) to identify nuclei.
As PLIN3 was the only perilipin to significantly increase in adipose tissue on the mRNA level, we measured the protein level of PLIN3 in primary adipocyte culture. We found virtually nonexistent levels of PLIN3 in four out of five women with PCOS, while the expression of PLIN3 was present in controls (Fig. 4D). Exercise-induced protein expression of PLIN3 in those four women with PCOS, who had no protein expression of PLIN3 before exercise, and was increased compared with pre-exercise expression in the one woman with PCOS, who had PLIN3 protein expression present before exercise training (Fig. 4D).

Discussion

Our data highlight a previously unrecognized, potential role of PLIN3 in adipose tissue lipolysis in women with PCOS. We show for the first time that the expression of PLIN3, along with PLIN1 and PLIN5, is greatly reduced in the adipose tissue of women with PCOS when compared with females matched for age, body composition, and metabolic phenotype. PLIN3 mRNA expression is increased – the only perilipin protein to significantly increase – following 16-week aerobic exercise training in women with PCOS. In addition, stromal-derived primary adipose cultures from PCOS women revealed that virtually no PLIN3 protein is expressed before exercise training, but becomes expressed following exercise training coupled with increases in oleate oxidation. We previously reported that exercise training in this cohort increased adipose tissue lipolysis under adrenergic stimulation (24). Our data suggest that PLIN3 may in part contribute to enhanced adipose tissue lipolytic stimulation.

The exercise benefits associated with improving the symptomatology of PCOS has been thoroughly investigated. Studies have shown improvements for women with PCOS in insulin resistance (19, 20, 35, 36), serum lipids (35, 37), and cardiovascular disease risk (19, 20, 35, 38). In addition, menstrual cycle improvements have been shown following exercise intervention in women with PCOS (19, 35, 39). Though some studies show reductions in body weight with exercise (19, 20, 35), several studies have shown that benefits occur without weight loss (28, 36, 40) and suggest that exercise for PCOS would be recommended even if weight loss were not achieved. Our study showed improvements in insulin resistance as measured by euglycemic–hyperinsulinemic clamps (Table 1) and improvements in menstrual function (28).

However, despite all these benefits of exercise, few studies have investigated molecular targets in the adipose tissue from women with PCOS. This seems important given the reported defects in adipose tissue function previously shown in women with PCOS (reviewed in (7)). We previously reported that 16 weeks of aerobic exercise can
improve basal and catecholamine-stimulated lipolysis of adipose tissue (24). A prior investigation showed a single-nucleotide polymorphism found in women with PCOS for the perilipin gene (25), indicating a potential for defects in the perilipin family of proteins, which are involved in lipolysis. In this study, we have investigated targets that regulate adipose tissue lipolysis, with an emphasis on PLIN3 given the upregulation of gene expression seen in the adipose tissue with exercise.

Studies centered on the perilipin family of proteins have focused heavily on the involvement of PLIN1 in the regulation of lipolysis in adipose tissue (reviewed in (41)) via a coupling of PLIN1 phosphorylation and ATGL activation (42, 43). However, the possible role of PLIN3 regulation in adipose lipolysis has been for the most part overlooked. Studies have shown PLIN3 to be highly expressed in adipocytes and described to have a preference for PLIN3 to coat smaller lipid droplets (44). In addition, studies in HeLa cells have revealed a colocalization of ATGL to with PLIN3-coated lipid droplets during lipolytic stimulus (26, 45). Furthermore, a handful of studies investigating the role of PLIN3 in skeletal muscle lipolysis demonstrated that PLIN3 colocalizes to lipid droplets in rats during epinephrine and muscle contractile stimulation (46, 47). Work from our group has recently shown that PLIN3 expression is upregulated following aerobic exercise in skeletal muscle, upregulated in response to lipolytic stimulation in human primary skeletal myotube cultures, and is positively associated with both ex vivo skeletal muscle and in vivo whole body fat oxidation (27).

Our data, using both in vivo and in vitro systems, show that the association between PLIN3 and lipolysis is evident in the adipose tissue of women with PCOS, indicating PLIN3 as a potential novel mediator of lipolysis.

Our data also highlight previously uninvestigated coatomer GTPases that might potentially be involved in the mediation of lipolysis. Our study shows a lower expression of ARF1, ARFRP1, and βCOP1 in women with PCOS compared with controls; the expression of these targets increases greatly following 16-week aerobic exercise training in the women with PCOS. Soni et al. (26) showed that ATGL was delivered to PLIN3-coated lipid droplets: a phenomenon that was inhibited when either treated with brefeldin A, a compound known to inhibit ARF1, or when several of the coatomer GTPases, such as ARF1, SEC23A, βCOP, and GBF1 were knocked down. Studies from our group have shown that ARF1, SEC23A, and ARFRP1 increase their expression with aerobic exercise in skeletal muscle and with lipolytic stimulation in primary skeletal muscle cultures (27). Finally, Guo et al. (48) found differential expression of coatomer GTPases with regards to differences in lipid droplet morphology. Likewise, we show that independent of differences in fat cell size, women with PCOS possess larger lipid droplet morphology when compared with their age- and body composition-matched controls. Lipid droplet morphology more closely resembled control females following 16 weeks of aerobic exercise in the women with PCOS. Alterations in these coatomer GTPases may perhaps be involved in the regulation of lipolysis not only as a mediator of lipase delivery to lipid droplets, but also as a regulator of lipid droplet morphology. Further functional investigations are warranted to determine these mechanisms.

We also observed reduced mRNA expression of lipases (both ATGL and MGL) in the adipose tissue of women with PCOS compared with controls. The expression of lipases increased with exercise, which would be expected given the increase in both in vivo and in vitro lipolysis. One aspect of lipase expression that remains elusive is that prior investigations have shown that testosterone mediates the expression of lipases (17, 18). Our data show that lipase expression is increased following exercise training with no decreases in circulating concentrations of total testosterone or FAI, perhaps indicating that aerobic exercise can increase adipose tissue lipolysis despite altering testosterone expression. Furthermore, we speculate that the increases in lipase expression we observed are not solely responsible for the increased lipolysis following exercise training. In fact, previous reports show that lipases do not act alone on lipid droplets to facilitate lipolysis, but require a chaperone such as the perilipin proteins (reviewed in (41)) or certain coatomer GTPases (26).

We recognize that our results and conclusions are based on observational data and associations between increases in lipolysis, and expression of PLIN3 and coatomer GTPases following exercise, and does not establish causative results. However, given the preponderance of our findings and data presented in prior investigations from our group and others, we believe that these associations are novel and relevant to highlight potentially new, unrecognized targets that may in part mediate adipose tissue lipolysis. In addition, it is noted that we only conducted the exercise intervention in the women with PCOS, because our original design was a prospective exercise intervention study for the women with PCOS. Although we did not conduct an exercise intervention in our control group because of later addition of the cross-section component, the goal of the study involving the control group was to perform the same set of state-of-the-art assessments (clamp, MRI, fat cell size, and DXA) and to obtain a control group
matched on the basis of metabolic phenotype. The cross-sectional comparison demonstrates novel and robust differences between the women with PCOS and controls and is able to further demonstrate that exercise in women with PCOS can rescue defects in lipolysis to levels observed in control subjects. Owing to our rigorous study design, supervised and carefully monitored exercise interventions, and extensive phenotyping analyses of the women with PCOS before and after their exercise intervention, we are confident that we were adequately able to identify the upregulation of PLIN3 gene expression as well as the coatamer GTPases, thus demonstrating their potential role in the improvements in adipose tissue lipolysis following exercise training. Further investigations would be necessary to understand specific mechanistic roles of PLIN3 in adipose tissue lipolysis.

In conclusion, based on prior evidence from single-nucleotide polymorphisms in the perilipin gene (25), we investigated the expression of the perilipin family of proteins in the adipose tissue from women with PCOS, and have shown that several perilipin proteins are differentially expressed in women with PCOS vs age- and body composition-matched control females. We have also shown that coatamer GTPases (ARF1, SEC23a, βCOP, GBF1, and ARFRP1) are differentially expressed in women with PCOS. A 16-week aerobic exercise training significantly increased PLIN3 expression as well as coatamer GTPases (ARF1, SEC23a, βCOP, GBF1, and ARFRP1). Stromal-derived primary adipose cultures showed increases in in vitro lipolysis, oleate oxidation, and reductions in triglyceride content following exercise training. In addition, adipose cultures revealed virtually no PLIN3 protein expression before exercise, which was then increased and became expressed following exercise training. Finally, primary adipose cultures demonstrated a large lipid droplet morphology, which was altered by exercise training, despite the fact that there were no differences in fat cell size from adipose tissue cross-sectionally. These data highlight previously unrecognized and novel potential targets that might be responsible for improving exercise-mediated lipolysis in the adipose tissue of women with PCOS.

Author contribution statement
J D Covington conceived the experiment, performed experiments, analyzed data, and wrote the manuscript; S Bajpeyi contributed to discussion and reviewed/edited the manuscript; C Moro performed experiments and reviewed/edited the manuscript; Y D Thoukakova performed experiments and reviewed/edited the manuscript; P J Ebenezer performed experiments and reviewed/edited the manuscript; D H Burk performed experiments and reviewed/edited the manuscript; E Ravussin contributed to discussion, conceived of the experiment, and reviewed/edited the manuscript; L M Redman provided helpful discussion, conceived the experiment, and reviewed/edited the manuscript. All authors gave final approval of the manuscript before submission. J D Covington is the guarantor of this work, and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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