Metformin Treatment May Increase Omentin-1 Levels in Women With Polycystic Ovary Syndrome

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OBJECTIVE—Polycystic ovary syndrome (PCOS) is associated with the metabolic syndrome. Decreased omentin-1 levels are associated with obesity and diabetes. To study the effects of metformin treatment on omentin-1 levels in PCOS subjects and effects of omentin-1 on in vitro migration and angiogenesis.

RESEARCH DESIGN AND METHODS—Serum omentin-1 was measured by ELISA. Angiogenesis was assessed by studying capillary tube formation in human microvascular endothelial cells (HMEC-1) on growth factor reduced Matrigel. Endothelial cell migration assay was performed in a modified Boyden chamber. Nuclear factor-κB (NF-κB) was studied by stably transfecting HMEC-1 cells with a *cis*-reporter plasmid containing luciferase reporter gene linked to five repeats of NF-κB binding sites. Akt phosphorylation was assessed by Western blotting.

RESULTS—Serum omentin-1 was significantly lower in PCOS women ($P < 0.05$). After 6 months of metformin treatment, there was a significant increase in serum omentin-1 ($P < 0.01$). Importantly, changes in hs-CRP were significantly negatively correlated with changes in serum omentin-1 ($P = 0.036$). In vitro migration and angiogenesis were significantly increased in serum from PCOS women ($P < 0.01$) compared with matched control subjects; these effects were significantly attenuated by metformin treatment ($P < 0.01$) plausibly through the regulation of omentin-1 levels via NF-κB and Akt pathways. CRP and VEGF induced in vitro migration, and angiogenesis was significantly decreased by omentin-1.

CONCLUSIONS—Increases in omentin-1 levels may play a role but are not sufficient to explain the decreased inflammatory and angiogenic effects of sera from metformin-treated PCOS women.
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Polycystic ovary syndrome (PCOS) is a proinflammatory state associated with type 2 diabetes, visceral obesity, and cardiovascular complications, features of the metabolic syndrome (1–5). The metabolic syndrome is associated with accumulation of visceral adipose tissue. Visceral adipose tissue produces cytokines termed "adipokines" implicated in the pathogenesis of diabetes and atherosclerosis (6–9).

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Omentin-1 has been described as a novel adipokine preferentially produced by visceral adipose tissue. In vitro experiments revealed that treatment with recombinant omentin-1 enhances insulin-stimulated glucose uptake in human adipocytes. Also, omentin-1 was shown to trigger Akt signaling in both the absence and presence of insulin (10,11). Additionally, omentin plasma levels and omentin gene expression in visceral adipose tissue are decreased in obesity (12). Recently, we have reported novel data showing a significant decrease of adipose tissue and circulating omentin-1 levels in overweight PCOS women (13). Thus, given the above functions of omentin-1, increasing omentin-1 levels in PCOS women may alleviate cardiometabolic dysfunction in these women.

We studied the effects of metformin treatment on omentin-1 levels in PCOS subjects and effects of omentin-1 and serum on in vitro migration and angiogenesis. Researchers have used serum to perform functional experiments in endothelial, cardiac, and neural cells (14–16). Finally, given the link between inflammation and angiogenesis (17), we explored nuclear factor-kappaB (NF-κB), Erk1/2, and Akt pathways, important regulators of inflammation and angiogenesis (18,19).

RESEARCH DESIGN AND METHODS

All PCOS patients met all three criteria of the revised 2003 Rotterdam European Society of Human Reproduction and Embryology (ESHRE)/American Society of Reproductive Medicine (ASRM) PCOS Consensus Workshop Group diagnostic criteria. The three criteria are *1*) oligo- and/or anovulation, *2*) clinical and/or biochemical signs of hyperandrogenism, and *3*) polycystic ovaries (20). Furthermore, all subjects in the control arm had normal findings on pelvic ultrasound scan, regular periods, and no hirsutism/acne. The control group had no discernible cause for infertility (unexplained infertility). No women were amenorrheic. All subjects that were studied did not have endometriosis. Exclusion criteria for the study included age > 40 years, known cardiovascular disease, thyroid disease, neoplasms, current smoking, diabetes, hypertension (blood pressure > 140/90 mmHg), and renal impairment (serum creatinine > 120 μmol/l). None of these women were on any medications for at least 6 months prior to the study, including oral contraceptives, glucocorticoids, ovulation induction agents, antidiabetic and antiobesity drugs, and estrogenic, antiandrogenic, or antihypertensive medication. All patients underwent anthropometric measurements. Blood pressure was measured in a sitting position within a quiet and calm environment after a rest of at least 5 min. The average of three measurements was obtained. Subjects were outpatients of the Department of Reproductive Medicine and Gynaecological Endocrinology of Magdeburg University and the Department of Obstetrics and Gynaecology of Martin-Luther-University Halle. The metabolic study was performed in the Outpatient Department of Endocrinology and Metabolism of Magdeburg University. Blood samples were collected between 0800 and 0900 h, after a 3-day normal carbohydrate diet and an overnight fast. Blood samples were drawn into serum separator tubes that contain no additives or anticoagulants, allowed to clot, centrifuged (3,000 rpm for 10 min) to separate sera, and stored at −80°C.

A treatment with metformin in an "off-label use" was offered to all PCOS women independently from the results of insulin sensitivity testing. In those PCOS women that agreed, therapy was initiated after basal assessment, and the dose of metformin was increased to a maintenance dose of 850 mg twice daily. All patients underwent anthropometric measurements before and after

metformin treatment. Carotid intima media thickness (IMT) was measured in PCOS subjects, before and after metformin (see below). A total of 83 women of Caucasian origin with PCOS were recruited, 49 of which did not participate in the metformin arm of the study. The baseline characteristics of the 49 PCOS women were comparable to the 34 PCOS women who participated in the metformin arm of the study. Furthermore, the 21 women who completed the metformin arm of the study were comparable to the 13 PCOS women who did not complete the metformin arm of the study. For the purposes of elucidating the effects of metformin in PCOS women, the 21 PCOS subjects were studied. The study design was approved by the Local Research Ethics Committee of the University of Magdeburg, and written informed consent was obtained from all participants, in accordance with the guidelines in The Declaration of Helsinki 2000.

Intima media thickness. Measurement of carotid IMT is a widely accepted tool to provide information about preclinical vascular disease. B-mode sonography of the proximal part of the carotid bulb was conducted on both sides, and the segments of the common carotid arteries 1.0 cm proximal were scanned longitudinally with Hitachi EUB-5000 plus-G (Hitachi Ltd., Tokyo, Japan) using a 10-MHz linear-array transducer. All women were investigated in a supine position with the head slightly hyperextended and turned away from the side being scanned. The image was focused on the posterior wall, and the resolution function was used to magnify the arterial far wall. When an optimal image was obtained, it was frozen, and the distance from the junction of the lumen and intima and the junction of the media and adventitia was measured by electronic calipers in end-diastolic phase, to minimize variability during the cardiac cycle. All images were photographed. Five measurements were conducted on each side, and the mean IMT was calculated as the average of these measurements. All measurements were performed by an experienced ultrasound sonographer (intra-observer coefficient of variation was 6.8%) who was blinded to the diagnosis of subjects.

Biochemical and hormonal analysis. Assays for glucose, insulin, cholesterol, triglycerides, luteinizing hormone, follicular stimulating hormone, testosterone, and rostenedione, dehydroepiandrosterone-sulfate, and sex hormone–binding globulin were performed using an automated analyzer (Abbott Architect, Abbott Laboratories, Abbott Park, USA). The estimate of insulin resistance by homeostasis model assessment (HOMA-IR) score was calculated as previously described (21). High-sensitivity C-reactive protein (hs-CRP) was determined immunoturbidimetrically using a modular system random-access analyzer (Roche Diagnostics). Omentin-1 levels in sera were measured using a commercially available ELISA kit (AXXORA, Nottingham, U.K.), according to manufacturer's protocol, with an intra-assay coefficient of variation of <6%.

Immunodepletion of serum omentin-1 and CRP. Immunodepletion of serum omentin-1 and CRP were performed using the immunoprecipitation protocol provided by Dr. Kazuyuki Itoh and Montecucco et al., respectively (14,22). One ml of human serum was incubated with mouse-anti-human omentin-1 IgG-protein G-Sepharose conjugate (1–2 μg/100–500 μg of total protein) or control mouse IgG-protein G-Sepharose conjugate; goat-anti-human CRP IgA-protein A-Sepharose conjugate (10 μg/100–500 μg of total protein) or control goat IgA-protein A-Sepharose conjugate, mixed overnight at 4°C and centrifuged to sediment the conjugated Sepharose beads. The supernatants were collected, and similar procedures were repeated eight times. Supernatants and sera were subjected to immunoblotting for omentin-1 as previously described (13). Immunoblotting for CRP is described below. Immunoblotting confirmed that these protocols decreased serum omentin-1 and CRP by ~80% (data not shown), respectively.

Endothelial cell culture. Human microvascular endothelial cells (HMEC-1) were obtained from the Center for Disease Control, Atlanta, USA HMEC-1 cells were cultured in MCDB medium (Sigma-Aldrich, Gillingham, U.K.) supplemented with 10% fetal calf serum (Sigma-Aldrich, Gillingham, U.K.), 100 IU/ml penicillin (Sigma-Aldrich, Gillingham, U.K.), 100 μg/ml streptomycin (Sigma-Aldrich), 5 ml of 200 mmol/l L-glutamine/500 ml of media, hydrocortisone 2 μmol/l, and epidermal growth factor 2 ng/ml (Invitrogen, Paisley, U.K.) at 37°C in 5% CO_2/95% air. Prior to each experiment, cells were fed with MCDB with addition of 1% fetal calf serum for 16 h.

For endothelial cell signaling experiments, HMECs were preincubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) for 30 min followed by treatment with or without 1% of human serum from normal (n = 39; individually tested) and PCOS women [before (n = 21; individually tested) and after (n = 21; individually tested) 6 months of metformin treatment] at different time points (0, 2, 5, 10, 20, 30, and 60 min). Dose- and time-dependent experiments were performed to determine the optimum concentration and time point.

In vitro angiogenesis assay. Angiogenesis was assessed by studying the formation of capillary tubelike structures by culturing HMEC-1 cells on growth factor reduced Matrigel (BD Biosciences, San Jose, USA). Matrigel was coated onto the culture plates as per manufacturer's instructions. HMEC-1 cells were preincubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) and/or with or without PI3K/Akt inhibitor (LY294002) (Calbiochem, San Diego, USA) for 30 min and 1 h, respectively, followed by treatment with or without 1% of human serum from normal (n = 39; individually tested) and PCOS women [before (n = 21; individually tested) and after (n = 21; individually tested) 6 months of metformin treatment] or CRP or omentin-1 [PCOS after metformin (n = 21; individually tested)] and CRP [PCOS before metformin (n = 21; individually tested)] immunodepleted sera. Vascular endothelial growth factor (VEGF) served as positive control. Trypsinized HMEC-1 cells were then seeded onto the Matrigel-coated plates at a density of 4–5 × 10^3/well in fresh media and incubated at 37°C. Dose- and time-dependent experiments were performed to determine the optimum concentration and time point. Capillary tube formation images were captured with a digital microscope camera system (Olympus, Tokyo, Japan). Image Pro Plus software was used to quantify tube length formation; the lengths of tubes in 3–4 randomly selected fields in each of the wells were measured (23–26).

In vitro migration assay. Endothelial cell migration assay was performed in a modified Boyden chamber using a protocol obtained from BD BioCoat Angiogenesis System (BD Biosciences, San Jose, USA). Endothelial cells were trypsinized; a cell suspension of 4 × 10^5 cells/ml was prepared, 250 μl of which was added to each of the trans-well inserts. Starvation media (750 μl) was added to the lower chamber and incubated overnight. After this, the cells were labeled by incubating with Hank's balanced salt solution medium (Sigma-Aldrich, Gillingham, U.K.) containing 4 μg/ml Calcein-AM (BD Biosciences, San Jose, USA) for 90 min. The cells were preincubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) and/or with or without PI3K/Akt inhibitor (LY294002) (Calbiochem, San Diego, USA) for 30 min and 1 h, respectively, followed by treatment with or without 1% of human serum from normal (n = 39; individually tested) and PCOS women [before (n = 21; individually tested) and after (n = 21; individually tested) 6 months of metformin treatment] or CRP or omentin-1 [PCOS after metformin (n = 21; individually tested)] and CRP [PCOS before metformin (n = 21; individually tested)] immunodepleted sera. VEGF served as positive control. Dose- and time-dependent experiments were performed to determine the optimum concentration and time point. Cells were fixed with 2% formaldehyde. Fluorescence of migrated cells was read in a fluorescence plate reader with bottom reading capabilities at excitation/emission wavelengths of 494/517 nm. Only those labeled cells that have migrated through the pores of the membrane will be detected (23–26).

NF-κB activation. We studied NF-κB activation by stably transfecting HMEC-1 cells with a *cis*-reporter plasmid containing luciferase reporter gene linked to five repeats of NF-κB binding sites (pNF-κB-Luc; Stratagene, LA Jolla, USA). Multiple clones were selected for the analysis of NF-κB activation. HMEC-1 cells were preincubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) for 30 min followed by treatment with or without 1% of human serum from normal (n = 39; individually tested) and PCOS women [before (n = 21; individually tested) and after (n = 21; individually tested) 6 months of metformin treatment] or CRP or omentin-1 [PCOS after metformin (n = 21; individually tested)] and CRP [PCOS before metformin (n = 21; individually tested)] immunodepleted sera for 2 h. Cells were lysed, and luciferase activities were measured. Experiments were also performed with TK plasmid (Promega, Southampton, U.K.) as negative control and tumor necrosis factor α (TNF-α) as positive control. Dose- and time-dependent experiments were performed to determine the optimum concentration and time point.

Western blotting. Endothelial cells were lysed with SDS sample buffer (5 mol/l urea, 0.17 mol/l SDS, 0.4 mol/l dithiothreitol, and 50 mmol/l Tris-HCl, pH 8.0), mixed, sonicated, boiled, centrifuged (5,000 rpm for 2 min), and then stored at −80°C until use. Eighty μg of each sample, supernatants, and sera were subjected to SDS-PAGE (8% resolving gel) and transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% BSA for 2 h. The PVDF membranes were then incubated with polyclonal primary rabbit-anti-human antibody for phospho-Erk1/2 (Cell Signaling Technology Inc., Beverly, USA) (1:1,000 dilution) or polyclonal primary rabbit-anti-human antibody for phospho-Akt (Ser473) (Cell Signaling Technology Inc., Beverly, USA) (1:1,000 dilution) or monoclonal primary goat-anti-human antibody for CRP (Sigma-Aldrich, Gillingham, U.K.) (1:1,000 dilution) overnight at 4°C. The membranes were washed thoroughly for 60 min with Tris-buffered saline–0.1% Tween before incubation with the secondary anti-rabbit horseradish peroxidase-conjugated immunoglobulin (Dako, Ely, U.K.) (1:2,000) or secondary anti-goat horseradish peroxidase-conjugated immunoglobulin (Dako, Ely, U.K.) (1:2,000) for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL+; GE Healthcare, Little Chalfont, U.K.). For standardization, the same membranes were stripped and reprobed using polyclonal primary rabbit-anti-human antibodies for total Erk1/2 (Cell Signaling Technology Inc., Beverly, USA) [1:1,000 dilution] or total Akt (Cell Signaling Technology Inc., Beverly, USA) [1:1,000 dilution].

The densities were measured using a scanning densitometer coupled to scanning software Scion Image (Scion Corporation, Frederick, USA). Stan-

TABLE 1
Clinical, hormonal, and metabolic features of women with PCOS and control subjects

Variable	PCOS (n = 21)	Control subjects (n = 39)	Significance
Age (years)	28 (26.5–31.5)	28 (23–32)	NS
BMI (kg/m^2)	32.8 (29.8–36.5)	29.1 (27.3–34.1)	NS
WHR	0.82 (0.76–0.88)	0.81 (0.78–0.86)	NS
Glucose (mmol/l)	5.1 (4.7–5.5)	4.8 (4.4–5.1)	NS
Insulin (pmol/l)	70.0 (54.5–94.5)	41.0 (32.0–55.0)	$P < 0.01$
HOMA-IR	2.2 (2.0–3.0)	1.2 (1.0–1.7)	$P < 0.01$
Cholesterol (mmol/l)	4.9 (4.1–5.3)	4.3 (3.8–4.8)	$P < 0.05$
Triglycerides (mmol/l)	1.0 (0.8–1.7)	0.8 (0.6–1.1)	$P < 0.05$
LH (IU/l)	7.8 (4.7–11.0)	3.8 (2.7–5.4)	$P < 0.01$
FSH (IU/l)	5.2 (4.0–6.2)	5.7 (4.3–7.5)	NS
Testosterone (nmol/l)	1.8 (1.5–2.5)	1.2 (1.0–1.6)	$P < 0.01$
Androstenedione (nmol/l)	3.0 (2.3–3.8)	2.6 (1.9–3.6)	NS
DHEA-S (μmol/l)	4.3 (2.7–5.6)	6.1 (3.8–7.5)	$P < 0.01$
SHBG (nmol/l)	27.0 (20.5–41.0)	45.0 (34.0–59.0)	$P < 0.01$
FAI	6.6 (4.6–9.7)	3.0 (1.9–4.4)	$P < 0.01$
SBP (mmHg)	125.0 (120.0–130.0)	120.0 (110.0–120.0)	$P < 0.01$
DBP (mmHg)	80.0 (75.0–80.0)	75.0 (70.0–80.0)	$P < 0.01$
IMT (mm)	0.53 (0.48–0.58)	0.42 (0.40–0.44)	$P < 0.01$
hs-CRP (mg/l)	3.3 (2.4–5.5)	1.3 (0.5–2.5)	$P < 0.01$
Omentin-1 (ng/ml)	23.7 (20.0–27.9)	27.6 (25.6–29.4)	$P < 0.05$

Data are median (interquartile range). Group comparison by Mann–Whitney U test. Free androgen index (FAI) = T (nmol/l)/SHBG (nmol/l) × 100. NS = not significant; LH, luteinizing hormone; FSH, follicular stimulating hormone; DHEA-S, dehydroepiandrosterone-sulfate; SHBG, sex hormone–binding globulin; SBP, systolic blood pressure; DBP, diastolic blood pressure.

dard curves were generated to ensure linearity of signal intensity over the range of protein amounts loaded into gel lanes. Comparisons of densitometric signal intensities for phospho-Erk1/2, phospho-Akt, total Erk1/2, and total Akt were made only within this linearity range.

Statistics. Data were analyzed by Mann–Whitney U test or Kruskal–Wallis ANOVA (post hoc analysis: Dunn's test) according to the number of groups compared. Data are medians (interquartile range). Spearman rank correlation was used for calculation of associations between variables. If individual bivariate correlations achieved statistical significance, multiple regression analysis with omentin-1 as dependent variable was performed to test the joint effect of these parameters on omentin-1. $P < 0.05$ was considered significant.

RESULTS

Table 1 shows the anthropometric, biochemical, and hormonal data in all subjects. Insulin, HOMA-IR, cholesterol, triglycerides, luteinizing hormone, testosterone, free androgen index, systolic blood pressure, diastolic blood pressure, IMT, and hs-CRP were significantly higher whereas dehydroepiandrosterone-sulfate and sex hormone–binding globulin were significantly lower in PCOS women.

Serum omentin-1 levels were significantly lower in PCOS subjects compared with control subjects [23.7 (20.0–27.9) versus 27.6 (25.6–29.4) ng/ml; $P < 0.05$: Table 1]. Serum progesterone levels in all women confirmed follicular phase of the menstrual cycle.

Table 2 shows the effects of metformin treatment on

TABLE 2
Clinical, hormonal, and metabolic features of women with PCOS (n = 21) before and after metformin treatment

Variable	Before metformin	After metformin	Significance
Age (years)	28 (26.5–31.5)	28 (27.5–32.5)	NS
BMI (kg/m^2)	32.8 (29.8–36.5)	31.4 (28.2–35.1)	NS
WHR	0.82 (0.76–0.88)	0.80 (0.74–0.87)	$P < 0.05$
Glucose (mmol/l)	5.1 (4.7–5.5)	4.8 (4.4–4.9)	$P < 0.05$
Insulin (pmol/l)	70.0 (54.5–94.5)	61.0 (43.5–83.0)	NS
HOMA-IR	2.2 (2.0–3.0)	1.6 (1.3–2.2)	$P < 0.05$
Cholesterol (mmol/l)	4.9 (4.1–5.3)	5.0 (4.1–5.4)	NS
Triglycerides (mmol/l)	1.0 (0.8–1.7)	1.2 (1.0–1.9)	NS
Testosterone (nmol/l)	1.8 (1.5–2.5)	1.3 (1.1–1.8)	$P < 0.05$
Androstenedione (nmol/l)	3.0 (2.3–3.8)	2.8 (2.2–3.6)	NS
DHEA-S (μmol/l)	4.3 (2.7–5.6)	5.4 (3.6–6.7)	NS
SHBG (nmol/l)	27.0 (20.5–41.0)	29.0 (21.5–46.5)	NS
FAI	6.6 (4.6–9.7)	5.3 (3.1–6.6)	NS
SBP (mmHg)	125.0 (120.0–130.0)	120.0 (120.0–127.5)	NS
DBP (mmHg)	80.0 (75.0–80.0)	75.0 (75.0–80.0)	NS
IMT (mm)	0.53 (0.48–0.58)	0.47 (0.41–0.53)	$P < 0.05$
hs-CRP (mg/l)	3.3 (2.4–5.5)	3.1 (2.0–6.2)	NS
Omentin-1 (ng/ml)	23.7 (20.0–27.9)	59.2 (52.1–60.6)	$P < 0.01$

Data are median (interquartile range). Group comparison by Mann–Whitney U test. Free androgen index (FAI) = T (nmol/l)/SHBG (nmol/l) × 100. NS = not significant; DHEA-S, dehydroepiandrosterone-sulfate; SHBG, sex hormone–binding globulin; SBP, systolic blood pressure; DBP, diastolic blood pressure.

TABLE 3
Linear regression analysis of variables associated with changes in serum omentin-1 levels (before and after metformin treatment), Δomentin-1, in PCOS subjects ($n = 21$)

Variable	Simple		Multiple	
	r	P	β	P
BMI (kg/m^2)	−0.400	0.072		
WHR	−0.445	0.043	−0.189	0.338
Glucose (mmol/l)	−0.471	0.031	−0.038	0.906
Insulin (pmol/l)	−0.014	0.953		
HOMA-IR	−0.461	0.035	−0.149	0.611
Cholesterol (mmol/l)	0.100	0.667		
Triglycerides (mmol/l)	0.390	0.080		
Testosterone (nmol/l)	−0.126	0.586		
Androstenedione (nmol/l)	0.139	0.548		
DHEA-S (μmol/l)	−0.232	0.311		
SHBG (nmol/l)	0.131	0.570		
FAI	−0.200	0.385		
SBP (mmHg)	−0.222	0.333		
DBP (mmHg)	0.032	0.889		
IMT (mm)	−0.032	0.892		
hs-CRP (mg/l)	−0.665	<0.010	−0.526	0.036

Spearman rank correlation was used for calculation of associations between variables. If individual bivariate correlations achieved statistical significance, multiple regression analysis with omentin-1 as dependent variable was performed to test the joint effect of these parameters on omentin-1. Multiple regression analysis contained WHR, glucose, HOMA-IR, and hs-CRP. DHEA-S, dehydroepiandrosterone-sulfate; SHBG, sex hormone–binding globulin; FAI, free androgen index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

serum omentin-1 levels. Metformin treatment was started in 34 women with PCOS. Only 21 women completed the study and were investigated after 6 months of metformin treatment. Reasons for subjects not completing study 2 were nausea and gastrointestinal side effects ($n = 4$), pregnancies ($n = 5$), incompliance ($n = 2$), and loss of contact ($n = 2$).

After 6 months of metformin treatment, there was a significant increase in serum omentin-1 [23.7 (20.0–27.9) versus 59.2 (52.1–60.6) ng/ml; $P < 0.01$: Table 2]. Also, there were significant decreases in waist-to-hip ratio (WHR), testosterone, glucose, HOMA-IR, and IMT.

Correlation of omentin-1 with covariates. Spearman rank analyses demonstrated that serum omentin-1 levels were significantly negatively correlated with BMI, WHR, glucose, HOMA-IR, and hs-CRP ($P < 0.05$). However, when subjected to multiple regression analysis, none of these variables were significantly negatively correlated with serum omentin-1 levels ($P > 0.05$).

Furthermore, we analyzed the correlation between the change in serum omentin-1 levels before and after metformin therapy (Δomentin-1) and the changes (Δ) in other covariates (Table 3). We found that Δomentin-1 was significantly negatively associated with ΔWHR, Δglucose, ΔHOMA-IR, and Δhs-CRP. When subjected to multiple regression analysis, only Δhs-CRP was significantly negatively correlated with Δomentin-1 ($\beta = -0.526$; $P = 0.036$).

Effects of omentin-1 on in vitro migration and angiogenesis. Given the above, we studied the effects of omentin-1 on in vitro migration and angiogenesis. Capillary tube formation was optimal at 24 h, after which capillary tubes begin to disintegrate. In vitro migration and angiogenesis at 24 h was significantly increased when comparing sera of PCOS women to normal control subjects (Figs. 1 and 2; $^{\#\#}P < 0.01$). Also, in vitro migration and angiogenesis were significantly decreased in PCOS women after 6 months of metformin treatment (Figs. 1 and 2; $^{**}P < 0.01$) and when omentin-1 (200 ng/ml) or the PI3K/Akt inhibitor (LY294002; 10 μmol/l) were added to sera (Figs. 1 and 2; $^{*}P < 0.05$, $^{**}P < 0.01$). Furthermore, CRP (1ug/ml) and VEGF (10 ng/ml) induced in vitro migration, and angiogenesis was significantly decreased by omentin-1 (Figs. 1 and 2; $^{*}P < 0.05$).

Moreover, in vitro migration and angiogenesis were significantly decreased in CRP (PCOS before metformin) but not significantly altered by omentin-1 (PCOS after metformin) immunodepleted sera (Figs. 1 and 2; $P > 0.05$, $^{*}P < 0.05$).

Effects of omentin-1 on NF-κB activation in human microvascular endothelial cells. In HMEC-1 cells, NF-κB was significantly activated by sera from normal and PCOS women compared with control subjects (Fig. 3; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$). NF-κB was significantly activated by sera from PCOS women compared with normal women (Fig. 3; $^{**}P < 0.01$). Also, NF-κB activation was significantly decreased in PCOS women after 6 months of metformin treatment (Fig. 3; $^{**}P < 0.01$) and when omentin-1 (200 ng/ml) was added to sera (Fig. 3; $^{*}P < 0.05$). Furthermore, CRP (1 ug/ml) and TNF-α (10 ng/ml) induced NF-κB activation was significantly decreased by omentin-1 (Fig. 3; $^{*}P < 0.05$).

Moreover, NF-κB activation was significantly decreased in CRP (PCOS before metformin) but not significantly altered by omentin-1 (PCOS after metformin) immunodepleted sera (Fig. 3; $P > 0.05$, $^{*}P < 0.05$).

Effects of omentin-1 on Erk1/2 and Akt signaling pathways in human microvascular endothelial cells. In HMEC-1 cells, Erk1/2 and Akt pathways were significantly activated by sera from normal and PCOS women compared with control subjects (Fig. 4*A* and *B*; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$). Erk1/2 and Akt phosphorylation were significantly increased by sera from PCOS women compared with normal women (Fig. 4*A* and *B*; $^{**}P < 0.01$). Also, Erk1/2 and Akt activation were significantly decreased in PCOS women after 6 months of metformin treatment (Fig. 4*A* and *B*; $^{*}P < 0.05$, $^{**}P < 0.01$). Furthermore, Akt activation was significantly decreased when omentin-1 (200 ng/ml) was added to sera from PCOS women before metformin treatment (Fig. 4*B*; $^{*}P < 0.05$). However, there was no significant difference in Erk1/2 activation (Fig. 4*A*). The detected proteins for phosphorylated Erk1/2, total Erk1/2, phosphorylated Akt, and total Akthad apparent molecular weights of 44/42, 44/42, 60, and 60 kDa, respectively (Fig. 4*A* and *B*, inserts).

DISCUSSION

We confirm our previous data of decreased circulating omentin-1 in PCOS women. Also, serum omentin-1 levels were significantly negatively correlated with BMI, WHR, glucose, HOMA-IR, and hs-CRP. We report that metformin (6 months treatment; 850 mg twice daily) significantly increases serum omentin-1 levels with concomitant decreases in insulin resistance and carotid IMT in PCOS subjects. Although the change in serum omentin-1 levels were significantly negatively associated with changes in WHR, glucose, HOMA-IR, and hs-CRP, only the change of hs-CRP was significantly negatively correlated with the change of serum omentin-1 levels when subjected to multiple regression analysis. The possible implication is

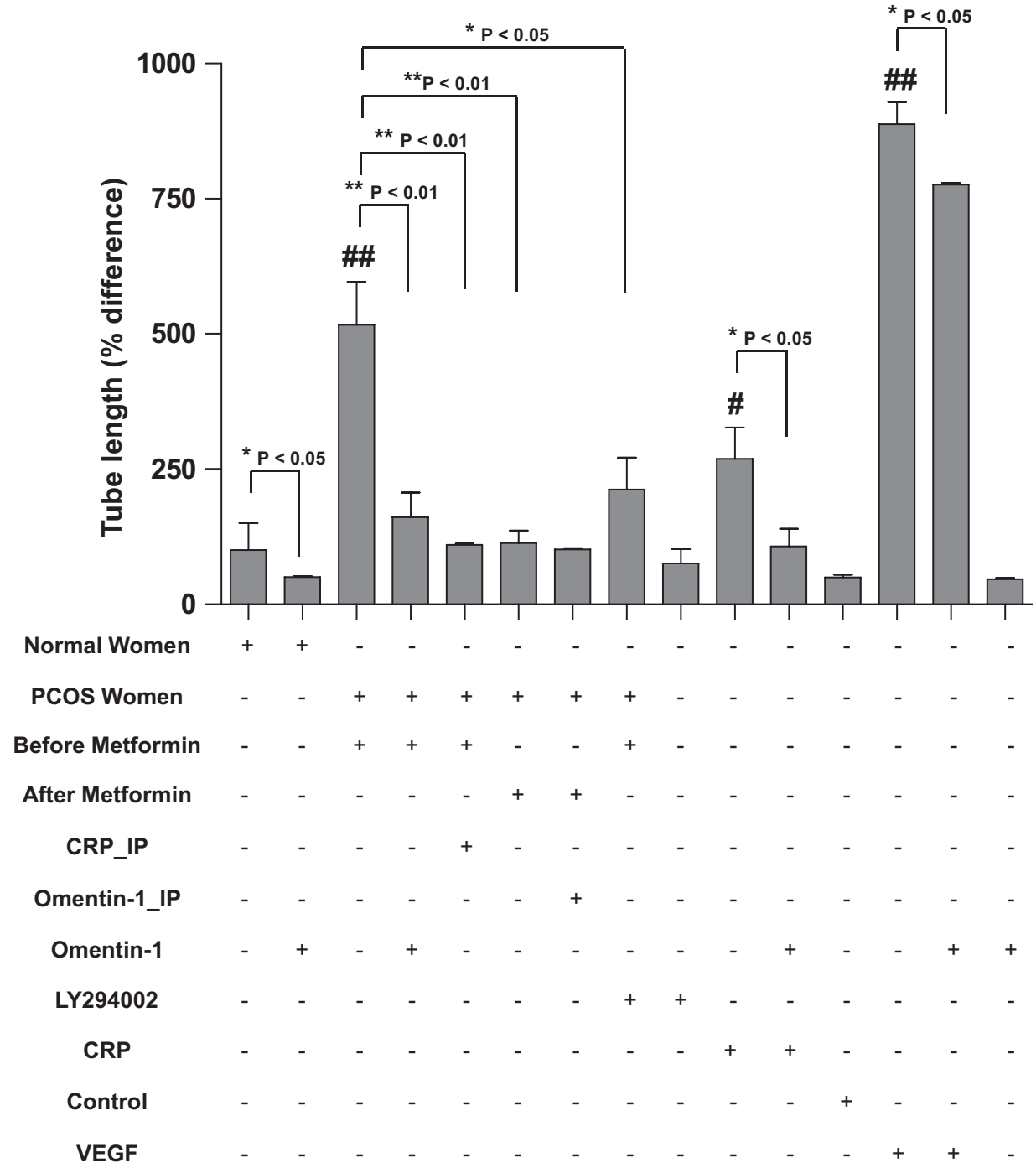

FIG. 1. In vitro tube formation by human serum at 24 h from normal control subjects (n = 39) with or without omentin-1 (200 ng/ml), PCOS women (n = 21) with or without omentin-1 (200 ng/ml) or PI3K/Akt inhibitor (LY294002; 10 μmol/l) or CRP immunodepletion (CRP_IP), PCOS women after 6 months of metformin treatment (n = 21) with or without omentin-1 immunodepletion (Omentin-1_IP), CRP (1ug/ml) with or without addition of omentin-1 (200 ng/ml) (n = 6), and VEGF (10 ng/ml) with or without addition of omentin-1 (200 ng/ml) (n = 6), respectively. Data are expressed as % difference of median of normal control subjects [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal–Wallis ANOVA (post hoc analysis: Dunn's test) and Mann–Whitney U test, respectively. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{*}P < 0.05$, $^{**}P < 0.01$.

that changes in omentin-1 levels associated with metformin treatment observed in our PCOS subjects are more robustly linked to changes in inflammatory rather than metabolic parameters. Of relevance, there is evidence that inflammatory markers other than CRP are elevated in PCOS subjects (3–5); also, studies have indicated metformin's potential anti-inflammatory effects via modulation of TNF-α, IL-6, IL-18, and IL-1-β levels (27–29).

In PCOS women, metformin corrects the associated endocrine and metabolic abnormalities (30); metformin counters IR by inhibiting hepatic glucose production (31–33). Therefore, glucose may regulate circulating omentin-1 levels; however, it is possible that the effect of metformin on omentin-1 levels could be via modulation of other circulating factors. Further studies are needed to clarify this point.

PCOS is a state of altered steroid milieu. We, like others

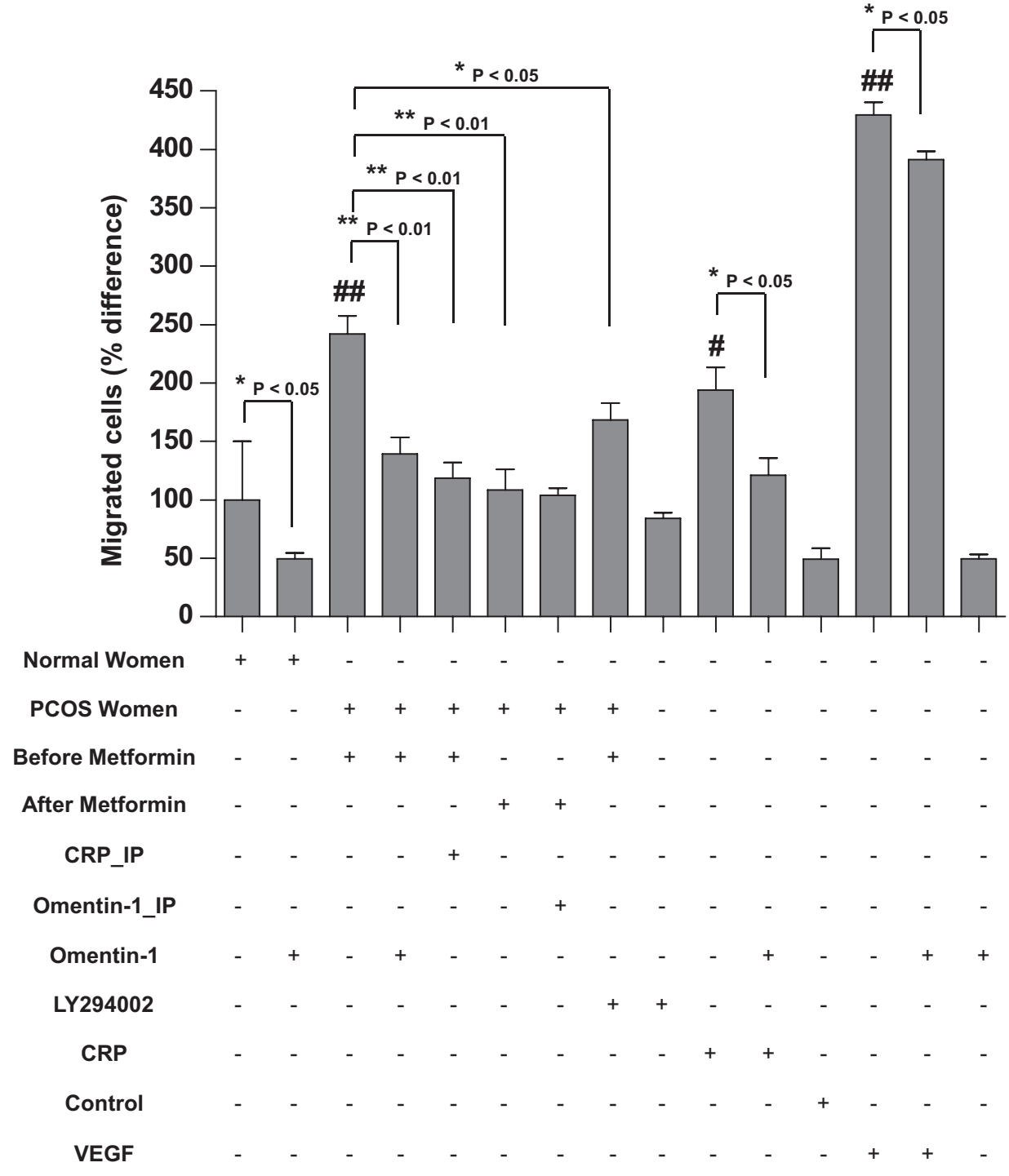

FIG. 2. Endothelial cell migration by human serum at 24 h from normal control subjects (n = 39) with or without omentin-1 (200 ng/ml), PCOS women (n = 21) with or without omentin-1 (200 ng/ml) or PI3K/Akt inhibitor (LY294002; 10 μmol/l) or CRP immunodepletion (CRP_IP), PCOS women after 6 months of metformin treatment (n = 21) with or without omentin-1 immunodepletion (Omentin-1_IP), CRP (1ug/ml) with or without addition of omentin-1 (200 ng/ml) (n = 6), and VEGF (10 ng/ml) with or without addition of omentin-1 (200 ng/ml) (n = 6), respectively. Data are expressed as % difference of median of normal control subjects [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal–Wallis ANOVA (post hoc analysis: Dunn's test) and Mann-Whitney U test, respectively. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{*}P < 0.05$, $^{**}P < 0.01$.

(34), observed a significant decrease in testosterone levels after metformin treatment. However, treatments of omental adipose tissue explants with gonadal and adrenal steroids revealed no meaningful effects on omentin-1 levels (13). It is therefore unlikely that the effects of metformin on serum omentin-1 levels observed in this study are attributable to altered gonadal and adrenal steroids.

Furthermore, functional assays revealed that in vitro migration and angiogenesis were significantly increased by sera from both normal and PCOS women; in PCOS women, in vitro migration and angiogenesis was attenuated after 6

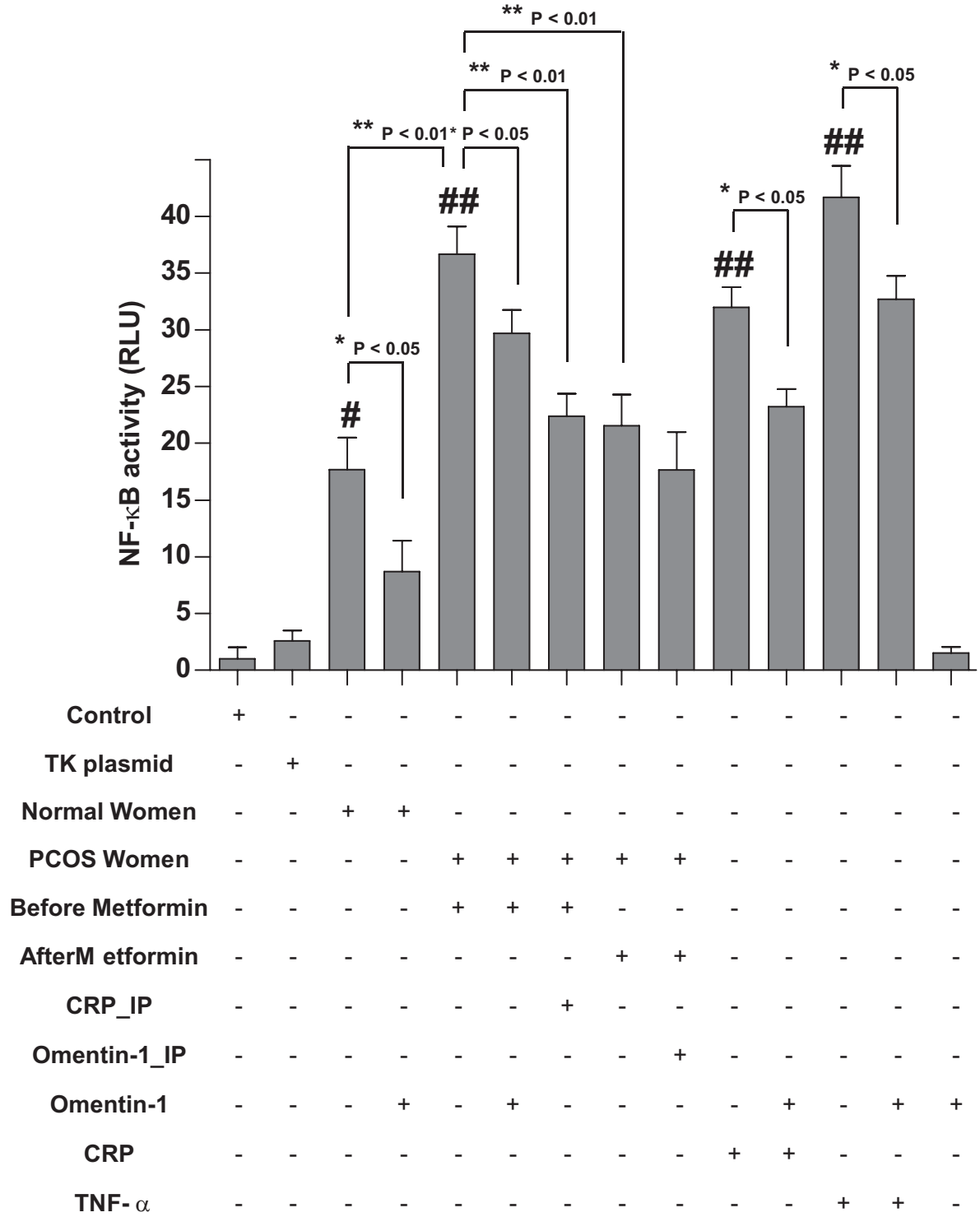

FIG. 3. Effects of serum from normal control subjects (n = 39) with or without omentin-1 (200 ng/ml), PCOS women (n = 21) with or without omentin-1 (200 ng/ml) or CRP immunodepletion (CRP_IP), PCOS women after 6 months of metformin treatment (n = 21) with or without omentin-1 immunodepletion (Omentin-1_IP), CRP (1ug/ml) with or without addition of omentin-1 (200 ng/ml) (n = 6), and TNF-α (10 ng/ml) with or without addition of omentin-1 (200 ng/ml) (n = 6), respectively, on NF-κB activation in HMEC-1 cells stably transfected with pNF-κB-luciferase at 2 h. Cells were lysed, and luciferase activities (RLU) were measured. Data are expressed as % difference of median of basal [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal–Wallis ANOVA (post hoc analysis: Dunn's test) and Mann–Whitney U test, respectively. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{*}P < 0.05$, $^{**}P < 0.01$.

months of metformin treatment. Addition of omentin-1 to sera from PCOS women before metformin treatment significantly decreased in vitro migration and angiogenesis. Omentin-1 also decreased the effects of CRP and VEGF induced in vitro migration and angiogenesis. However, in vitro migration and angiogenesis were significantly decreased in CRP but not significantly altered by omentin-1 immunodepleted sera. It is important to consider that omentin-1 levels in tissues are unknown; the levels are likely to be significantly higher than in sera as sequestration of omentin-1 in vascular tissue, in

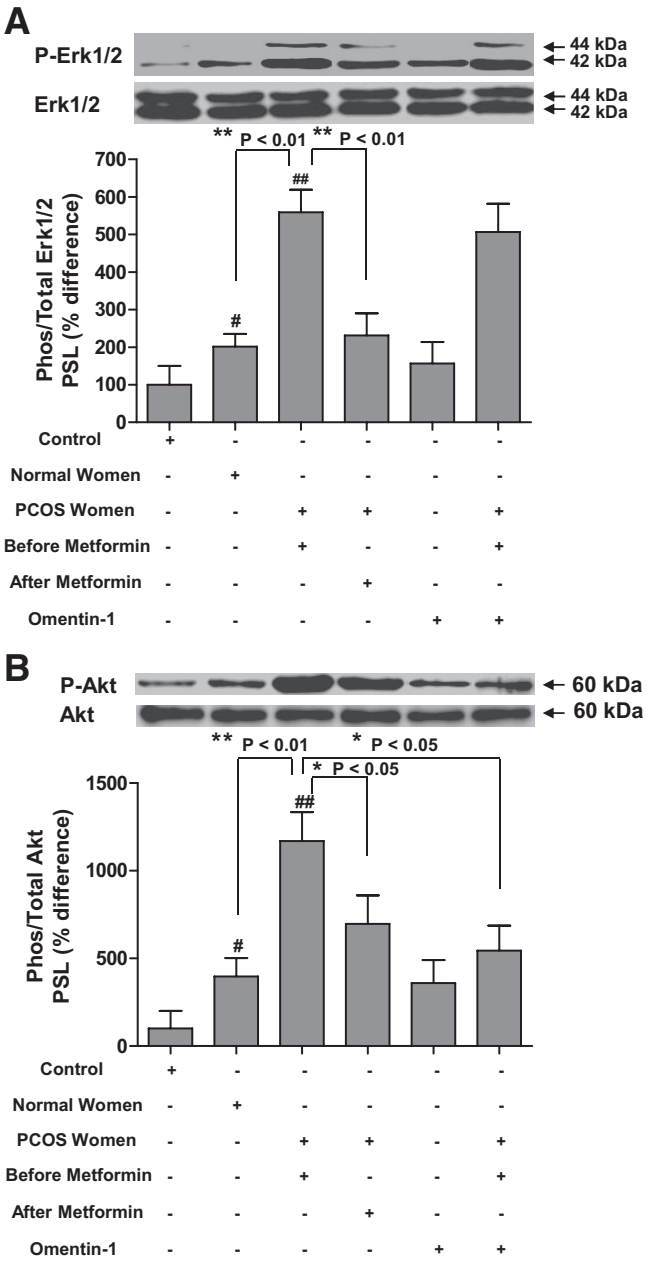

FIG. 4. Effects of serum from normal control subjects (n = 39), PCOS women (n = 21), and PCOS women after 6 months of metformin treatment with or without addition of omentin-1 (200 ng/ml) (n = 21) on Erk1/2 and Akt phosphorylation in HMEC-1 cells at 2 and 5 min, respectively. Data are expressed as % difference of median of basal [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal–Wallis ANOVA (post hoc analysis: Dunn's test) and Mann–Whitney U test, respectively. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{*}P < 0.05$, $^{**}P < 0.01$.

particular, within stromal-vascular cells, has been shown (10,35). All of these effects appear to be associated with NF-κB and Akt signaling pathways, both well-established mediators of angiogenesis (36–38). Taken together, increases in omentin-1 levels may play a role but are not sufficient to explain the decreased inflammatory and angiogenic effects of sera from metformin-treated PCOS women. It is important to bear in mind that metformin may also modulate angiogenesis via the VEGF system. Of relevance, metformin decreases VEGF levels in type 2 diabetic subjects (39).

The link between angiogenesis and the pathogenesis of atherosclerosis has been well described (17). Given our data of an increase in circulating omentin-1 levels with concomitant decrease in IMT, omentin-1 may have a potential role in the pathogenesis of atherosclerosis.

Omentin-1, by promoting activation of the Akt signaling pathway and in turn modulating the endothelial nitric oxide synthase in endothelial cells, may impact upon endothelial dysfunction, a step that is crucial in the pathogenesis of atherosclerosis (40,41). Our novel data of omentin-1 on angiogenesis in relation to the Akt signaling pathway thus clarifies omentin-1's potentially important role in the pathogenesis of cardiovascular disorders.

A limitation of our study may relate to the lack of a lean PCOS comparator group. Only 3 of the 21 PCOS subjects and 6 of the 39 control subjects had BMI < 25 kg/m^2; thus, no meaningful result could be obtained. Future studies should address this point. Furthermore, given ethical constraints, that is, blood samples could and were only obtained at baseline and after 6 months of metformin treatment, it is therefore difficult to be certain as to whether changes in serum omentin-1 levels precede or follow changes in clinical and hormonal indexes as a consequence of metformin treatment. Also, as diet and lifestyle modifications were only subjectively assessed, the changes in serum omentin-1 levels may be partially attributable to diet and lifestyle modifications. Further studies are needed to elucidate this point. Additionally, like others (42), we used WHR, a universally accepted, simple, and cost-effective measure of adiposity as a surrogate marker for central fat mass. However, Cascella et al. has reported that WHR does not distinguish between visceral and subcutaneous fat mass in PCOS women (43). Therefore, more accurate and equally cost-effective methods of assessing central obesity are needed. Finally, given that PCOS is a proinflammatory state associated with clustering of cardiovascular risk factors, it would be of importance to study the effect of metformin therapy in the context of omentin-1 biology on reactive oxygen species in PCOS women, as reactive oxygen species not only induces tissue damage and inflammation but also is increased in dysmetabolic states including PCOS (44).

In conclusion, we provide evidence that increases in omentin-1 levels may play a role but are not sufficient to explain the decreased inflammatory and angiogenic effects of sera from metformin-treated PCOS women.Our findings provide novel insights into the relationship between obesity, insulin resistance, and metformin therapy with dysregulated angiogenesis in PCOS women in the context of omentin-1 biology.

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B.K.T. researched data, contributed to discussion, wrote the manuscript, and reviewed/edited the manuscript. R.A. researched data, contributed to discussion, and reviewed/edited the manuscript. S.F. and J.C. researched data. H.L.

and H.S.R. contributed to discussion and reviewed/edited the manuscript.

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