Insulin and Metformin Regulate Circulating and Adipose Tissue Chemerin

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OBJECTIVE—To assess chemerin levels and regulation in sera and adipose tissue from women with polycystic ovary syndrome (PCOS) and matched control subjects.

RESEARCH DESIGN AND METHODS—Real-time RT-PCR and Western blotting were used to assess mRNA and protein expression of chemerin. Serum chemerin was measured by enzyme-linked immunosorbent assay. We investigated the in vivo effects of insulin on serum chemerin levels via a prolonged insulin-glucose infusion. Ex vivo effects of insulin, metformin, and steroid hormones on adipose tissue chemerin protein production and secretion into conditioned media were assessed by Western blotting and enzyme-linked immunosorbent assay, respectively.

RESULTS—Serum chemerin, subcutaneous, and omental adipose tissue chemerin were significantly higher in women with PCOS ($n = 14$; $P < 0.05$, $P < 0.01$). Hyperinsulinemic induction in human subjects significantly increased serum chemerin levels ($n = 6$; $P < 0.05$, $P < 0.01$). In adipose tissue explants, insulin significantly increased ($n = 6$; $P < 0.05$, $P < 0.01$) whereas metformin significantly decreased ($n = 6$; $P < 0.05$, $P < 0.01$) chemerin protein production and secretion into conditioned media, respectively. After 6 months of metformin treatment, there was a significant decrease in serum chemerin ($n = 21$; $P < 0.01$). Importantly, changes in homeostasis model assessment–insulin resistance were predictive of changes in serum chemerin ($P = 0.046$).

CONCLUSIONS—Serum and adipose tissue chemerin levels are increased in women with PCOS and are upregulated by insulin. Metformin treatment decreases serum chemerin in these women. *Diabetes* **58:**1971–1977, 2009

Polycystic ovary syndrome (PCOS), a common endocrinopathy affecting 5–10% of women in the reproductive age, is characterized by menstrual dysfunction and hyperandrogenism and is associated with insulin resistance and pancreatic β-cell dysfunction, impaired glucose tolerance (IGT), type 2 diabetes, dyslipidemia, and visceral obesity (1,2). The consequent hyperinsulinemia is more prevalent in lean and obese women with PCOS when compared with age- and weight-matched normal women (3).

The metabolic syndrome is associated with excessive accumulation of central body fat. As well as its role in energy storage, adipose tissue produces several hormones and cytokines termed 'adipokines' that have widespread effects on carbohydrate and lipid metabolism. They appear to play an important role in the pathogenesis of insulin resistance, diabetes, and atherosclerosis (4). Furthermore, it is apparent that accumulation of visceral adipose tissue poses a greater cardiometabolic risk than subcutaneous adipose tissue (5) as removal of visceral rather than subcutaneous adipose tissue has been shown to improve insulin sensitivity (6). Moreover, differences in gene expression of adipocyte-secreted molecules (adipokines) suggest that there are inherent adipose tissue depot–specific differences in the endocrine function of adipose tissue. In relation to this, we have published data on the increased levels of vaspin in women with PCOS (7); vaspin is a recently described adipokine mainly formed in human visceral adipose tissue that has insulin-sensitizing effects (8).

Recently, Bozaoglu et al. (9) reported chemerin as a novel adipokine, circulating levels of which significantly correlated with BMI, circulating triglycerides, and blood pressure, features of the metabolic syndrome. In addition, chemerin or chemerin receptor knockdown impaired differentiation of 3T3-L1 cells and attenuated the expression of adipocyte genes involved in glucose and lipid homeostasis (10).

With the aforementioned in mind and the fact that there is no literature with regards to chemerin in human adipose tissue and its regulation, in study 1, we assessed circulating chemerin as well as mRNA expression and protein levels of chemerin in subcutaneous and omental adipose tissue depots in women with PCOS against age, BMI, and waist-to-hip ratio (WHR) in matched control subjects. Furthermore, we studied the in vivo (study 2) and ex vivo effects of insulin on circulating chemerin levels via a prolonged insulin-glucose infusion in humans and primary adipose tissue explant cultures, respectively. In study 3 we studied the effects of metformin therapy, widely used in the treatment of PCOS in women, on circulating chemerin levels in tandem with associated changes to clinical, hormonal, and metabolic parameters in the same cohort of PCOS in women. Additionally, we studied the ex vivo effects of metformin and steroid hormones in human primary adipose tissue explants.

RESEARCH DESIGN AND METHODS

Study 1. Seventy three subjects were recruited consecutively from the infertility clinic in accordance with the inclusion/exclusion criteria (PCOS: $n = 19$; control subjects: $n = 54$) as previously described (7); 62 subjects were

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

	PCOS ($n = 14$)	Control subjects ($n = 14$)	Significance
Age (years)	29.5 (28–38)	32.5 (29–35)	NS
BMI (kg/m^2)	30.5 (27.8–30.9)	28.8 (28–30.5)	NS
WHR	0.89 (0.78–0.99)	0.84 (0.81–0.96)	NS
Glucose (mmol/l)	5.5 (4.8–6.0)	4.5 (4.3–5.2)	$P < 0.01$
Insulin (pmol/l)	78.9 (42.0–91.1)	57.3 (48.5–66.0)	NS
HOMA-IR	3.0 (2.1–3.6)	2.0 (1.4–2.2)	$P < 0.05$
Cholesterol (mmol/l)	5.1 (4.1–5.7)	5.0 (4.8–5.5)	NS
Triglycerides (mmol/l)	2.0 (1.5–2.3)	0.9 (0.7–1.4)	$P < 0.01$
Luteinizing hormone (IU/l)	7.0 (6.0–10.0)	6.0 (5.0–7.0)	NS
Follicle-stimulating hormone (IU/l)	6.0 (6.0–7.0)	6.5 (5.0–8.0)	NS
Prolactin (mU/l)	348.0 (305.0–387.0)	304.5 (211.0–322.0)	NS
E$_2$ (pmol/l)	353.0 (287.0–471.0)	174.5 (129.0–264.0)	$P < 0.01$
Progesterone (nmol/l)	1.6 (1.3–2.1)	2.2 (1.7–2.3)	NS
17-OH-P (nmol/l)	2.5 (2.1–2.8)	2.0 (1.2–2.3)	NS
Testosterone (nmol/l)	1.7 (1.5–2.1)	0.8 (0.6–0.9)	$P < 0.01$
Androstenedione (nmol/l)	15.6 (14.2–16.8)	6.3 (5.0–8.2)	$P < 0.01$
DHEA-S (µmol/l)	5.9 (5.4–6.6)	4.5 (4.0–5.3)	$P < 0.05$
SHBG (nmol/l)	31.5 (26.7–35.2)	59.0 (47.7–66.0)	$P < 0.01$
FAI	19.5 (14.5–21.2)	3.9 (3.3–6.1)	$P < 0.01$
Leptin (ng/ml)	24.9 (19.7–29.0)	24.1 (19.3–28.8)	NS
Adiponectin (µg/ml)	5.67 (4.52–7.5)	6.27 (5.52–9.48)	NS
Chemerin (ng/ml)	6.02 (5.96–6.04)	2.62 (1.96–3.23)	$P < 0.01$

Data are medians (interquartile range). Group comparison by Mann-Whitney U test. FAI = Testosterone (nmol/liter)/SHBG (nmol/liter) × 100. NS = not significant. FAI, free androgen index; SHBG, sex hormone–binding globulin.

from a previous study (7). Of the 19 PCOS subjects recruited, five withdrew before the study could be completed. In the control group, seven subjects did not complete the study. From the remaining 47 control subjects, 14 control subjects matched for age, BMI, and WHR were included in the final analysis (Table 1). Blood and adipose tissue samples were obtained as previously described (7). Subcutaneous adipose tissue was obtained from a 3 cm horizontal midline incision ~3 cm above the symphysis pubis. Omental adipose tissue was obtained by excisional biopsy from the greater omentum. The local research ethics committee of University Hospitals Coventry and Warwickshire NHS Trust approved the study, and all patients involved gave their informed consent in accordance with the guidelines in The Declaration of Helsinki 2000.

Study 2. We measured circulating chemerin in six (three men, three women) healthy subjects [age: (mean ± SD) 26.5 ± 8 years, BMI: 23.2 ± 2.5 kg/m^2]. All subjects studied were nonsmokers and otherwise healthy. Women volunteers had regular periods, no clinical or biochemical hyperandrogenism, and were not taking any medications. Exclusion criteria for the study, as previously described (7), included age over 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 medication for at least 6 months before the study, including oral contraceptives, glucocorticoids, ovulation induction agents, antidiabetic and antiobesity drugs, or estrogenic, antiandrogenic, or antihypertensive medication. The local research ethics committee of University Hospitals Coventry and Warwickshire NHS Trust approved the study, and all patients involved gave their informed consent in accordance with the guidelines in The Declaration of Helsinki 2000. To account for the possible diurnal variation in chemerin levels, we obtained a daily control curve by measuring fasting chemerin levels at 30-min intervals from 0800 to 1000 h. Subsequently, chemerin levels were measured at 2-h intervals until 2400 h and then at 0400 h as well as at 30-min intervals from 0800 to 1000 h on day 2. On the following day, the same subjects were subjected to a prolonged insulin-glucose infusion for 26 h beginning at 0800 h. Insulin (Human Actrapid) was administered intravenously as a priming dose of 0.04 units/kg followed by continuous infusion of 0.5 mU · kg^{-1} · min^{-1}. By choosing this rate of insulin infusion we expected to achieve hyperinsulinemia with an approximate four- to sixfold elevation of basal insulinemia (11). Fasting blood samples were drawn at 30-min intervals between 0800 and 1000 h on day 1 and day 2 of the prolonged insulin-glucose infusion (the first and the last 2 h of the infusion). Intermediate blood samples were taken at 2-h intervals until 2400 h and then at 0400 h on day 2. Glucose levels were maintained between 4.0 and 6.0 mmol/l.

Study 3. Subjects were recruited and blood samples were obtained as previously described (7). A treatment with metformin in an "off-label use"

was offered to all women with PCOS independently from the results of insulin sensitivity testing, as per standard clinical practice. 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.

Biochemical and hormonal analysis. Assays were performed using an automated analyzer as previously described (7). The estimate of insulin resistance by homeostasis model assessment (HOMA-IR) score was calculated as previously described (12). Circulating leptin and adiponectin levels were measured with a coated-tube immunoradiometric assay kit (Diagnostic Systems Laboratories, Sinsheim, Germany) and by a commercially available RIA kit (Millipore, Watford, U.K.), respectively, according to manufacturer's protocol.

Chemerin levels in sera and conditioned media from human adipose tissue explants were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Abingdon, U.K.), according to manufacturer's protocol, with an intra-assay coefficient of variation of less than 9%.

Primary explant culture. Adipose tissue organ explants were cultured using a protocol that was a modification of the method described by Fried and Moustaid-Moussa (13). Adipose tissue explants were cultured for 24 h with or without the addition of insulin, testosterone, 17β-estradiol, androstenedione, dehydroepiandrosterone-sulfate (DHEA-S), or metformin, as previously described (7).

Total RNA extraction and cDNA synthesis. Total RNA was extracted from adipose tissue samples and cDNA synthesized as previously described (7).

RT-PCR. Quantitative PCR of chemerin was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (7). The sequences of the sense and antisense primers used were chemerin (252 bp) 5′-AGACAAGCTGCCGGAAGAGG-3′ and 5′-TGGAGAAGGCGAACTGTCCA-3′; β-actin (216 bp) 5′-AAGAGAGGCATCCT-CACCCT-3′ and 5′-TACATGGCTGGGGTCTTGAA-3′.

Western blotting. Protein lysates were prepared as previously described (7). Protein samples (30 µg/lane) containing SDS sample buffer (5 M urea, 0.17 M SDS, 0.4 M dithiothreitol, and 50 mmol/l Tris-HCl, pH 8.0) were subjected to SDS-PAGE (10% resolving gel) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). PVDF membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% BSA for 2 h. The PVDF membranes were then incubated with polyclonal primary goat anti-human antibody for chemerin (R & D Systems) (1:1,000 dilution) or monoclonal primary rabbit anti-human antibody for β-actin (Cell Signaling Technology, Beverly, CA) (1:1,000 dilution) overnight at 4°C. The membranes were washed thoroughly for 60 min with TBS 0.1% Tween before incubation with the

secondary anti-goat horseradish peroxidase–conjugated immunoglobulin (Dako, Ely, U.K.) (1:2,000) or secondary anti-rabbit horseradish peroxidase–conjugated immunoglobulin (Dako) [1:2000], respectively, for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL+; GE Healthcare, Little Chalfont, U.K.). Human chemerin protein (R & D Systems) was used as positive control and water as negative control (data not shown).

Statistics. Data were analyzed by Student's t test, Mann-Whitney U test, Kruskal-Wallis, or Friedman's ANOVA (post hoc analysis: Dunn's test) according to the number of groups compared; $P < 0.05$ were considered significant. For Western immunoblotting experiments, the densities were measured using a scanning densitometer coupled to scanning software Scion Image (Scion Corporation, Frederick, MD). Standard 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 chemerin and β-actin were made only within this linearity range. Spearman Rank correlation was used for calculation of associations between variables; $P < 0.05$ was considered significant.

RESULTS

Demographic data. Table 1 shows the anthropometric, biochemical, and hormonal data in all subjects. Glucose, HOMA, triglycerides, 17β-estradiol (E_2), testosterone, androstenedione, DHEA-S levels, and free androgen index were significantly higher, whereas sex hormone–binding globulin was significantly lower in women with PCOS.

Serum chemerin levels were significantly higher in PCOS subjects than in control subjects (6.02 [5.96–6.04] vs. 2.62 [1.96–3.23] ng/ml; $P < 0.01$; Table 1). Serum progesterone levels in all women confirmed follicular phase of the menstrual cycle.

mRNA expression and protein levels of chemerin in normal and PCOS women. We detected chemerin mRNA in adipose tissue of all subjects, and subsequent sequencing of the PCR products confirmed gene identity. Real-time RT-PCR analysis corrected over β-actin showed a significant increase of chemerin expression in subcutaneous (*$P < 0.05$) and omental (**$P < 0.01$) adipose tissue of PCOS when compared with normal control subjects (Fig. 1A). However, no significant difference in chemerin mRNA expression was observed when comparing corresponding omental with subcutaneous adipose tissue in PCOS and normal subjects (Fig. 1A; $P > 0.05$). Changes noted at mRNA level were also reflected at protein level (Fig. 1B).

Study 2: Effects of a prolonged insulin-glucose infusion on serum chemerin levels. Insulin infusion resulted in elevation of fasting insulinemia from 78.1 ± 12.0 pmol/l to 294.6 ± 31.0 pmol/l. Insulin levels remained elevated until the end of the prolonged insulin-glucose infusion (366.0 ± 37.0 pmol/l). Chemerin levels remained unaltered throughout the control day from 2.06 ± 0.08 ng/ml between 0800 and 1000 h to 1.92 ± 0.07 ng/ml between 0800 and 1000 h the next day (Fig. 2; $P > 0.05$).

There was a profound effect of insulin on chemerin levels over 26 h of insulin infusion: from 2.54 ± 0.32 ng/ml between 0800 and 1000 h to 3.97 ± 0.37 ng/ml between 0800 and 1000 h the following day (Fig. 2; *$P < 0.05$). The increase in chemerin levels was relatively acute approaching maximal values at 4 h (Fig. 2; 5.08 ± 0.27 ng/ml, **$P < 0.01$) and persisting throughout the entire period of hyperinsulinemia.

Study 3: Effects of metformin treatment on serum chemerin 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. The anthropometric, biochemical, and hormonal
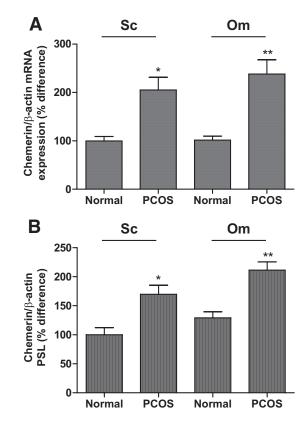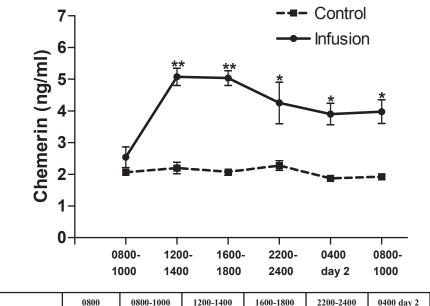
FIG. 1. A: Chemerin mRNA expression relative to β-actin was significantly increased in human subcutaneous (Sc) and omental (Om) adipose tissue depots when comparing PCOS women ($n = 14$) with normal control subjects ($n = 14$), using real-time RT-PCR. Data are expressed as percent difference of median of human subcutaneous adipose tissue of normal control subjects. Each experiment was carried out in three replicates. Group comparison by Kruskal-Wallis ANOVA and post hoc Dunn's test; *$P < 0.05$, **$P < 0.01$. B: Densitometric analysis of chemerin immune complexes having normalized to β-actin revealed that protein levels of chemerin were significantly increased in human subcutaneous and omental adipose tissue depots, respectively, when comparing all women with PCOS with all normal control subjects. Data are expressed as percent difference of median of normal control subjects. Each experiment was carried out in three replicates. Group comparison by Kruskal-Wallis ANOVA (post hoc analysis: Dunn's test). *$P < 0.05$, **$P < 0.01$; PSL, phosphostimulated light units.

data of PCOS subjects investigated in study 1 were not significantly different compared with the PCOS subjects investigated in study 3. Reasons for subjects not completing study 3 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 were significant decreases in serum chemerin, WHR, E_2, testosterone, glucose, and HOMA-IR (Table 2).

Dose-dependent effects of insulin, testosterone, 17β-estradiol, androstenedione, DHEA-S, and metformin on chemerin protein production and secretion into conditioned media from control human omental adipose tissue explants. We found that chemerin protein production and secretion into conditioned media was significantly increased dose dependently by insulin in control human omental adipose tissue explants (Fig. 3; *$P < 0.05$ and **$P < 0.01$, respectively). Interestingly, metformin significantly decreased chemerin protein production and secretion into conditioned media in control human omental adipose tissue explants (Fig. 4 and *$P < 0.05$ and **$P < 0.01$, respectively). Similar results were

Time	0800	0800-1000	1200-1400	1600-1800	2200-2400	0400 day 2	0800-1000
Insulin (pmol/L)	78.1 ± 12.0	276.3 ± 25.1	294.6 ± 31.0	345.4 ± 31.7	365.1 ± 38.1	339.7 ± 32.5	366.0 ± 37.0
10% Glucose Infusion (ml/h)	-	116.2 ± 11.9	120.4 ± 8.9	122.5 ± 11.4	125.8 ± 9.3	138.3 ± 11.7	128.3 ± 13.2
Glucose (mmol/L)	4.3 ± 0.5	4.8 ± 0.6	4.3 ± 0.7	5.6 ± 0.9	5.2 ± 0.5	4.4 ± 1.0	4.7 ± 0.8

FIG. 2. Mean concentrations of chemerin in nanogram per milliliter in all subjects, before and after insulin infusion. Insulin infusion resulted in elevation of fasting insulinemia from 78.1 ± 12.0 pmol/l to 294.6 ± 31.0 pmol/l. Insulin levels remained elevated until the end of the prolonged insulin-glucose infusion (366.0 ± 37.0 pmol/l). Data are means ± SD. Group comparison by Student's t test. *$P < 0.05$, **$P < 0.01$.

observed in control human subcutaneous adipose tissue explants (data not shown). With respect to gonadal and adrenal steroids, no significant effects on chemerin protein production and secretion were observed (data not shown).
Association of chemerin with covariates. In study 1, Spearman's rank analyses demonstrated that serum and subcutaneous and omental adipose tissue chemerin levels were significantly positively associated with BMI, WHR, glucose, insulin, HOMA-IR, and circulating triglycerides ($P < 0.05$). However, when subjected to multiple regression analysis, none of these variables were predictive of serum chemerin levels ($P > 0.05$).

In study 3, we analyzed the correlation between the change in serum chemerin levels before and after metformin therapy (Δchemerin) and the changes (Δ) in other covariates (Table 3). ΔChemerin was significantly positively associated with ΔWHR, Δglucose, Δinsulin, ΔHOMA-IR, and Δtriglycerides. When subjected to multiple regression analysis with WHR, glucose, insulin, HOMA-IR, and triglycerides, only HOMA-IR was predictive of changes in serum chemerin levels (Table 3), whereas other models of multiple regression analysis revealed no other significant predictors of changes in serum chemerin levels (see supplemental data, available in an online appendix at

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

	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)	$P < 0.01$
Insulin (pmol/l)	70.0 (54.5–98.0)	60.0 (43.5–81.0)	NS
HOMA-IR	2.1 (1.7–3.1)	1.6 (1.3–2.3)	$P < 0.05$
Cholesterol (mmol/l)	4.9 (4.1–5.3)	5.0 (4.0–5.4)	NS
Triglycerides (mmol/l)	1.0 (0.7–1.9)	1.2 (1.0–1.7)	NS
E$_2$ (pmol/l)	329.8 (164.9–494.7)	207.1 (103.6–310.7)	$P < 0.05$
Testosterone (nmol/l)	1.8 (1.4–2.2)	1.3 (1.0–1.8)	$P < 0.05$
Androstenedione (nmol/l)	10.9 (8.0–14.0)	9.7 (7.6–12.4)	NS
DHEA-S (μmol/l)	4.4 (2.8–5.8)	5.4 (3.6–6.7)	NS
SHBG (nmol/l)	27.0 (21.0–41.0)	25.0 (20.5–46.5)	NS
FAI	6.2 (4.6–8.0)	5.2 (3.1–6.6)	NS
Leptin (ng/ml)	26.5 (20.7–30.9)	25.1 (17.9–30.0)	NS
Adiponectin (μg/ml)	4.85 (4.14–6.88)	3.69 (2.91–5.55)	NS
Chemerin (ng/ml)	6.36 (5.80–6.83)	2.19 (2.04–4.02)	$P < 0.01$

Data are medians (interquartile range). Group comparison by Mann-Whitney U test. FAI = Testosterone (nmol/liter)/SHBG (nmol/liter) × 100. NS = not significant. FAI, free androgen index; SHBG, sex hormone–binding globulin.
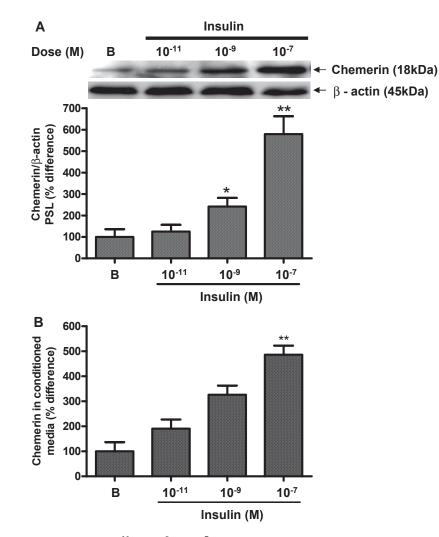
A

Insulin

Dose (M)   B 10^{-11} 10^{-9} 10^{-7}

← Chemerin (18kDa)

← β - actin (45kDa)

FIG. 3. *A*: Dose-dependent effects of insulin (10^{-11} M, 10^{-9} M, 10^{-7} M) in the presence of 5 mmol/l D-glucose on chemerin net protein production in control human omental adipose tissue explants at 24 h were assessed by Western blotting. Western blot analysis of protein extracts from omental adipose tissue demonstrate that the antibody against chemerin and the antibody against β-actin recognized bands with apparent molecular weights of 18 kDa and 45 kDa, respectively (Fig. 3*A*, *inserts*). Densitometric analysis of chemerin immune complexes having normalized to β-actin, respectively, revealed that protein levels of chemerin were significantly increased by insulin (10^{-9} M, 10^{-7} M) in control human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison by Friedman's ANOVA and post hoc Dunn's test. *$P < 0.05$, **$P < 0.01$. *B*: Dose-dependent effects of insulin (10^{-11} M, 10^{-9} M, 10^{-7} M) in the presence of 5 mmol/l D-glucose on chemerin secretion into conditioned media from control human omental adipose tissue after 24 h were measured by ELISA. Chemerin secretion was significantly increased (by 10^{-9} M, 10^{-7} M) from control human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison by Friedman's ANOVA and post hoc Dunn's test. *$P < 0.05$, **$P < 0.01$.

http://diabetes.diabetesjournals.org/cgi/content/full/db08-1528/DC1).

DISCUSSION

We present novel data showing a significant increase of serum and subcutaneous and omental adipose tissue chemerin mRNA expression as well as protein levels in women with PCOS. More importantly, we demonstrate the potent and robust regulation of chemerin in vivo and ex vivo by insulin as well as its modulation by metformin treatment. Unfortunately, because of technical limitations in adipose tissue procurement, we were unable to obtain sufficient amounts of sample/patient tissue to perform stromal vascular separation in adipose tissue depots. We could not account for a potential adipocyte hypertrophy–related side effect. These limitations not withstanding, adipose tissue from our PCOS women, compared with control subjects, express more chemerin.

PCOS have an increased incidence of IGT and type 2 diabetes (1,2). The higher serum and adipose tissue chemerin levels in women with PCOS is of interest given that it has recently been reported that IGT and type 2 diabetes *Psammomys obesus* animals (a unique polygenic animal model for obesity and type 2 diabetes) had higher adipose tissue chemerin levels than normal glucose–tolerant *Psammomys obesus* animals. In the same study, significant positive associations with BMI and circulating triglycerides in normal glucose–tolerant human subjects were noted (9). However, no data exists on chemerin and its regulation in human adipose tissue. In our study, we found significant positive associations between circulating chemerin as well as chemerin levels in subcutaneous and omental adipose tissue with BMI, WHR, glucose, insulin, HOMA-IR, and circulating triglycerides. However, it is unlikely that either BMI or WHR are responsible for these findings, as both groups were matched for these variables.
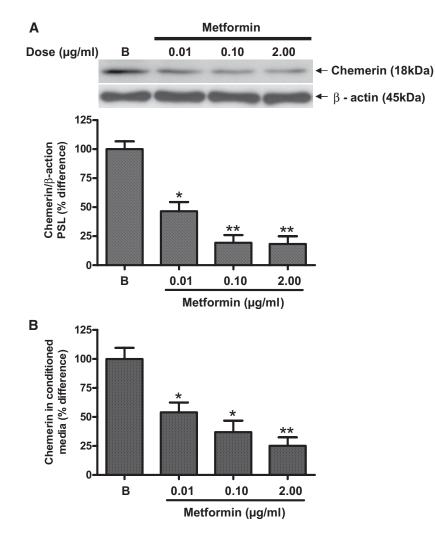
FIG. 4. *A*: Dose-dependent effects of metformin (0.01, 0.1, and 2.00 µg/ml) in the presence of 5 mmol/l D-glucose on chemerin net protein production in control human omental adipose tissue explants at 24 h were assessed by Western blotting. Western blot analysis of protein extracts from omental adipose tissue demonstrate that the antibody against chemerin and the antibody against β-actin recognized bands with apparent molecular weights of 18 kDa and 45 kDa, respectively (Fig. 4*A*, *inserts*). Densitometric analysis of chemerin immune complexes having normalized to β-actin, respectively, revealed that protein levels of chemerin were significantly decreased by metformin (0.01, 0.1, and 2.00 µg/ml) in control human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison by Friedman's ANOVA and post hoc Dunn's test. *$P < 0.05$, **$P < 0.01$. *B*: Dose-dependent effects of insulin (0.01, 0.1, and 2.00 µg/ml) in the presence of 5 mmol/l D-glucose on chemerin secretion into conditioned media from control human omental adipose tissue after 24 h were measured by ELISA. Chemerin secretion was significantly decreased (by 0.01, 0.1, and 2.00 µg/ml) from control human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison by Friedman's ANOVA and post hoc Dunn's test. *$P < 0.05$, **$P < 0.01$.

In addition, caution needs to be exercised as these correlations may be spurious, without causative significance, resulting from the simple fact that our PCOS women had higher levels for all these parameters.

Importantly, in study 2 we derive novel observations of a profound increase in chemerin levels by insulin in vivo. This effect of insulin appears to be relatively acute, achieving a maximal effect 4 h after commencement of insulin and persisting throughout the entire period of hyperinsulinemia. Furthermore, this is in agreement with our data on the regulation of chemerin protein production ex vivo. It is important to bear in mind that the regulation of chemerin in adipose tissue is probably multifactorial. Moreover, it would be of interest to know whether or not the effects of insulin on chemerin production are also applicable to other tissues given our in vivo data. Future studies are needed to elucidate the role of other factors that regulate chemerin production. Taken together, the above findings could tentatively explain the increased

levels of chemerin seen in our insulin-resistant PCOS subjects. Of secondary interest, there appears to be no circadian variation in chemerin levels as depicted in Fig. 2. It should be emphasized that the primary aim of this study was not to investigate the circadian variation of chemerin. In addition, our study utilized relatively small numbers of subjects because of the challenge imposed by the prolonged insulin clamp study; hence, care needs to be exercised in interpretation of these results.

More importantly, in study 3 we report for the first time that metformin (6 months treatment; 850-mg twice daily) significantly decreases circulating chemerin levels with a concomitant decrease in insulin resistance in PCOS subjects. Additionally, although the change in serum chemerin levels were significantly positively associated with changes in WHR, glucose, insulin, HOMA-IR, and triglycerides, when subjected to multiple regression analysis only HOMA-IR was predictive of serum chemerin levels. Taken together, we hypothesize that elevated chemerin

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

	Simple		Multiple	
	Estimate	P	Estimate	P
ΔBMI (kg/m^2)	0.156	0.500	—	—
ΔWHR	0.486	0.026	0.293	0.329
ΔGlucose (mmol/l)	0.510	0.018	−0.338	0.234
ΔInsulin (pmol/l)	0.503	0.020	−0.387	0.205
ΔHOMA-IR	0.772	<0.010	0.628	0.046
ΔCholesterol (mmol/l)	0.276	0.226	—	—
ΔTriglycerides (mmol/l)	0.490	0.024	0.456	0.107
ΔE$_2$ (pmol/l)	0.240	0.327	—	—
ΔTestosterone (nmol/l)	−0.130	0.576	—	—
ΔAndrostenedione (nmol/l)	0.039	0.867	—	—
ΔDHEA-S (μmol/l)	−0.055	0.814	—	—
ΔSHBG (nmol/l)	0.236	0.304	—	—
ΔFAI	−0.224	0.330	—	—
ΔLeptin (ng/ml)	−0.003	0.989	—	—
ΔAdiponectin (μg/ml)	0.026	0.911	—	—

In multiple linear regression analysis, values included were WHR, glucose, insulin, HOMA-IR, and triglycerides. FAI, free androgen index; SHBG, sex hormone–binding globulin.

levels may be a compensatory mechanism to insulin resistance in our cohort of PCOS subjects. Furthermore, of relevance, an elegant study by Bozaoglu et al. (9) describes important findings that circulating chemerin levels in type 2 diabetes human subjects were not significantly higher than those in normal control subjects. Therefore, our observations are timely as they may explain the findings by Bozaoglu et al. (9), given that quite probably a proportion of their type 2 diabetic study subjects may have been taking metformin (Bozaoglu et al. had not reported the medications taken by their type 2 diabetic subjects, with metformin arguably being the most common first-line oral hypoglycemic therapy to treat type 2 diabetes employed by most physicians in both developing as well as developed countries. Therefore, our study highlights metformin therapy as a confounding factor concerning the regulation of circulating chemerin levels. This should alert investigators who are studying chemerin biology to consider this in their analyses. In addition, this point may also apply to other forms of antidiabetic therapy; hence, caution needs to be exercised appropriately.

A limitation of our study may relate to the number of subjects studied. However, obtaining BMI/WHR-matched and menstrual cycle–synchronized blood and adipose tissue samples from two sites impeded subject recruitment. Our observations are highly consistent and significant and raise interesting questions on the mechanisms regulating chemerin production. Moreover, a sample size as in our study is only likely to detect differences that are enormous/significant. Finally, it should be emphasized that our findings relate only to overweight women with PCOS, and

it would be of interest to perform this study with lean women with PCOS.

In conclusion, we report novel findings of a significant increase of circulating and adipose tissue chemerin, a novel adipokine, in women with PCOS as well as the potent and robust regulation of chemerin by insulin in vivo and ex vivo. More importantly, we present novel data that metformin treatment significantly decreases circulating chemerin levels in women with PCOS. The physiologic and pathologic significance of our findings remain to be further elucidated.

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