

# Chemerin Is a Novel Adipokine Associated with Obesity and Metabolic Syndrome

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Soluble protein hormones are key regulators of a number of metabolic processes, including food intake and insulin sensitivity. We have used a signal sequence trap to identify genes that encode secreted or membrane-bound proteins in *Psammomys obesus*, an animal model of obesity and type 2 diabetes (T2D). Using this signal sequence trap, we identified the chemokine chemerin as being a novel adipokine. Gene expression of chemerin and its receptor, chemokine-like receptor 1 (CMKLR1), was significantly higher in adipose tissue of obese and type 2 diabetic *P. obesus* compared with lean, normoglycemic *P. obesus*. Fractionation of *P. obesus* adipose tissue confirmed that chemerin was predominantly expressed in adipocytes, whereas CMKLR1 was expressed in both adipocytes and stromal-vascular cells of adipose tissue. In 3T3-L1 adipocytes,

chemerin was markedly induced during differentiation, whereas CMKLR1 was down-regulated during differentiation. Serum chemerin levels were measured by ELISA in human plasma samples from 114 subjects with T2D and 142 normal glucose tolerant controls. Plasma chemerin levels were not significantly different between subjects with T2D and normal controls. However, in normal glucose tolerant subjects, plasma chemerin levels were significantly associated with body mass index, circulating triglycerides, and blood pressure. Here we report, for the first time, that chemerin is an adipokine, and circulating levels of chemerin are associated with several key aspects of metabolic syndrome. (*Endocrinology* 148: 4687–4694, 2007)

SECRETED PROTEINS regulate a wide range of processes in key metabolic tissues, such as liver, skeletal muscle, adipose tissue, pancreas, and the brain. Anabolic secreted protein hormones such as insulin play essential roles in the storage of energy after food intake by promoting glucose uptake in skeletal muscle and adipose tissue, and decreasing glucose production from the liver (1). In contrast to insulin, the catabolic hormone glucagon is typically produced in times of fasting, and promotes the breakdown of energy stored as glycogen in muscle and liver, and increases gluconeogenesis in the liver. The sensitivity of insulin-responsive tissues to insulin action is also regulated by secreted proteins, e.g. the cytokine TNF- $\alpha$  induces insulin resistance in muscle and adipose tissue, whereas adiponectin and IL-15 increase insulin sensitivity (2–4).

Adipose tissue has been identified as an important endocrine organ that not only stores energy but also regulates energy homeostasis and metabolism (5). It communicates with liver, skeletal muscle, and the brain via secreted protein hormones (adipokines). These adipokines have diverse roles and may regulate long-term energy balance (e.g. leptin) or insulin sensitivity of insulin responsive tissues (e.g. adi-

ponectin and resistin) (6). Recently, it has been observed that elevated adiposity associated with obesity induces inflammation in adipose tissue (7, 8). This process is characterized by the elevated expression of the proinflammatory cytokine, monocyte chemoattractant protein-1, in adipose tissue and subsequent accumulation of activated macrophages. These macrophages secrete other proinflammatory cytokines such as TNF- $\alpha$  and IL-6, which can act in a paracrine fashion to affect insulin sensitivity of adipocytes or as endocrine factors to affect insulin sensitivity of distal tissues, such as skeletal muscle and liver (5). More recently, adipose tissue has influenced insulin sensitivity via nonhormone secretory factors such as retinol binding protein 4 (RBP4), the major transporter of retinoic acid in the body (9, 10). Elevated levels of RBP4 have been found in mice and humans that are obese and have type 2 diabetes (T2D), and mice engineered to overexpress RBP4 have exhibited insulin resistance (10, 11).

To identify novel secreted proteins (and their receptors) that play a role in metabolism, we developed a signal sequence trap (SST) method to recognize secreted proteins expressed by *Psammomys obesus*, a unique polygenic animal model for obesity and T2D. We have demonstrated, using this SST and *in vitro* and *in vivo* model systems, that the chemokine chemerin is an adipokine that exhibits increased expression in adipose tissue from obese *P. obesus*. We have measured circulating chemerin levels in human subjects, and shown that plasma chemerin concentrations are strongly associated with body mass index (BMI), plasma triglycerides, and blood pressure. These findings suggest that chemerin may play an important role in obesity and metabolic syndrome.

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Abbreviations: BMI, Body mass index; CMKLR1, chemokine-like receptor 1; FBS, fetal bovine serum; IGT, impaired glucose tolerant; mL-3, murine IL-3; NGT, normal glucose tolerance; PBST, PBS 0.05% Tween 20; RBP4, retinol binding protein 4; SST, signal sequence trap; T2D, type 2 diabetes; WHR, waist hip ratio.

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## Materials and Methods

### Human samples

All plasma samples were obtained during the course of large-scale epidemiological studies of obesity, diabetes, and metabolic syndrome from Mauritius. Mauritius is a subtropical island located in the South Western Indian Ocean with a population of about 1.3 million. An estimated 70% of the population is of Asian Indian origin (54% Hindu and 16% Muslim), 2% are of Chinese origin, and 28% are of the “general” population, which mainly comprises people with mixed African and Malagasy ancestry, with some European and Indian admixture (Creoles). The three ethnic groups each demonstrate a high prevalence of obesity and diabetes (12, 13). The samples consisted of 256 individuals, of whom 142 had normal glucose tolerance (NGT), and 114 had T2D. Samples were randomly selected between individuals who had an age between 35 and 65 yr. All samples were obtained with informed consent, and all protocols were approved by the Inner Eastern Health Care Network Institution review board.

### Experimental animals

A colony of *P. obesus* was maintained at Deakin University, Geelong, Australia. Animals were fed *ad libitum* a standard laboratory diet comprising 63% carbohydrate, 25% protein, and 12.5% fat (Baristoc, Pakenhams, Australia). They were housed in low-top cages, maintained in a temperature controlled room ( $22 \pm 1^\circ\text{C}$ ) with a 12–12 h light-dark cycle (0600–1800 h light). At 16 wk of age, animals were classified into three groups according to their blood glucose and plasma insulin concentrations, as previously described (14). NGT animals were lean and had normal glucose tolerance, impaired glucose tolerant (IGT) animals were obese and had impaired glucose tolerance, and T2D animals were obese and had T2D. Whole blood glucose was measured using an enzymatic glucose analyzer (model 27; Yellow Springs Instruments, Yellow Springs, OH), and plasma insulin concentrations were determined using a double antibody solid phase RIA kit (Phadaseph; Kabi Pharmacia Diagnostics, Uppsala, Sweden). At 18 wk of age, males were separated into two treatment groups, either “fed,” in which animals have access to chow *ad libitum*, or “fasted,” whereby animals were fasted for 24 h. Animals were killed by anesthetic overdose (pentobarbitone, 120 mg/kg; Sigma-Aldrich, St. Louis, MO), and tissue was excised and snap frozen in liquid nitrogen. Samples were stored at  $-80^\circ\text{C}$  until RNA was extracted. Deakin University Animal Welfare Committee approval was granted for *P. obesus* tissue to be collected and used for gene characterization. All experiments were conducted according to strict National Health and Medical Research Council, and Deakin University Animal Welfare Committee guidelines.

### RNA extraction and quantification

Total RNA was extracted from *P. obesus* tissues, and from 3T3-L1 adipocytes using TRIzol (Invitrogen Corp., Carlsbad, CA) reagent and purified using RNeasy columns as per the manufacturer's instructions (QIAGEN, Cologne, Germany). The quality and quantity of the RNA was determined using the Agilent Bioanalyzer and RNA 600 Nano Assay kit (Agilent Technologies, Palo Alto, CA).

### SST

The SST methodology used to identify chemerin will be described in detail elsewhere. Briefly, mRNA from *P. obesus* liver was used to generate cDNA using a random nonamer primer with a *Not*I restriction site at the 5' end and the Superscript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning kit (Invitrogen). This cDNA was cloned into *Xho*I and *Not*I sites in the retrovirus packaging vector pLNCX2 (Clontech, Palo Alto, CA). The cDNA was cloned upstream of murine IL-3 (mIL-3) that was engineered to lack its signal peptide and encoded amino acids 26–166 (accession no. NP\_034686). The cDNA library was transfected into Plat-E retrovirus packaging cell line using Lipofectamine PLUS (Invitrogen). After 48 h, the virus-containing supernatant was used to infect mIL-3-dependent FDPC-1 cells overnight in the presence of  $8 \mu\text{g/ml}$  polybrene and  $1 \text{ ng/ml}$  mIL-3. The next day, the cells were washed, resuspended in media lacking mIL-3, and seeded into 96-well round bottom plates. In the absence of mIL-3, only cells

infected with a virus containing a cDNA encoding a signal sequence should secrete mIL-3 and proliferate. After 7–10 d in culture, genomic DNA was extracted from clones that grew in the absence of mIL-3 using a DNeasy 96 tissue kit (QIAGEN). A nested PCR protocol was used to amplify cDNAs using the extracted genomic DNA. The PCR products were purified using the ArrayIt 384 well PCR Purification kit (ArrayIt Microarray Technology, TeleChem Intl., Inc., Sunnyvale, CA). Purified PCR products were sequenced using BigDye Terminator Mix v3.1 reagents (Applied Biosystems, Foster City, CA).

### RT-PCR

cDNA was prepared by RT-PCR using the Superscript III Reverse Transcription for RT-PCR kit (Invitrogen). The reaction mix was then incubated in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) at  $50^\circ\text{C}$  for 60 min,  $85^\circ\text{C}$  for 5 min, and  $4^\circ\text{C}$  for 5 min.

### Relative quantification of gene expression using real-time PCR

Gene expression levels were quantitated by SYBR Green real-time PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Real-time PCR was performed using a SYBR Green master mix kit (Applied Biosystems). The PCR conditions were  $50^\circ\text{C}$  for 2 min,  $95^\circ\text{C}$  for 10 min, and 40 cycles of  $95^\circ\text{C}$  for 15 sec and  $60^\circ\text{C}$  for 1 min. Relative gene expression was calculated as  $2^{-\Delta\text{Ct}}$ . Real-time PCR primers were: *P. obesus* chemerin forward 5'-TGGGCCTTCCGAGAGATG-3'; *P. obesus* chemerin reverse 5'-AGACGACCACACAGGTCACGTA-3'; *P. obesus* chemokine-like receptor 1 (CMKLR1) forward 5'-AGCTTTGACCGCTGCATCTC-3'; *P. obesus* CMKLR1 reverse 5'-GGAAGCAAGAA-GAAAGCCAAGAG-3'; mouse chemerin forward 5'-CCAAGTCCCAAG-GAAGGA-3'; mouse chemerin reverse 5'-CGCCTTCTCCGTTGGT-3'; mouse CMKLR1 forward 5'-TGGCCGACTTCTGTTCAC-3'; and mouse CMKLR1 reverse 5'-CCCGAACACCCAGTGGTAGT-3'.

### Fractionation of adipose tissue

Approximately 2 g mesenteric adipose tissue was minced and washed several times with Krebs-Ringer phosphate buffer [ $12.5 \text{ mM}$  HEPES,  $120 \text{ mM}$  NaCl,  $1.2 \text{ mM}$   $\text{MgSO}_4$ ,  $1 \text{ mM}$   $\text{CaCl}_2$ ,  $0.4 \text{ mM}$   $\text{NaH}_2\text{PO}_4$ ,  $0.6 \text{ mM}$   $\text{Na}_2\text{HPO}_4$ ,  $6 \text{ mM}$  KCl,  $5 \text{ mM}$  glucose, and 3% BSA fraction V (pH 7.4)] to remove any dead cells, connective tissue, or blood cells. The tissues were digested in  $0.75 \text{ mg/ml}$  collagenase type I (Worthington Biochemical Corp., Lakewood, NJ) in a shaking water bath ( $37^\circ\text{C}$ ) for approximately 20 min. The digested samples were filtered through a nylon mesh into 25 ml fresh Krebs-Ringer phosphate buffer and centrifuged at  $300 \times g$ , 10 min at room temperature. The floating adipocyte layer was collected and the supernatant aspirated to reveal the pellet fraction. Both fractions were snap frozen in liquid nitrogen and stored  $-80^\circ\text{C}$  for subsequent RNA extraction.

### Tissue culture

3T3-L1 adipocytes were grown in high-glucose DMEM (25 mM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) at  $37^\circ\text{C}$ , 10%  $\text{CO}_2$ . Cells were passaged 1:10 into six-well plates (Life Technologies, Inc., Gaithersburg, MD). Two days after confluence, differentiation of fibroblasts into adipocytes was initiated by the addition of high-glucose DMEM, 10% FBS, 50 U/ml penicillin and 50  $\mu\text{g/ml}$  streptomycin, 0.5 mM 1-methyl-3-isobutylxanthine (Sigma-Aldrich), 2.5  $\mu\text{M}$  dexamethasone (Sigma-Aldrich), and 0.166 U/ml insulin (Humulin, 100 U/ml; Eli Lilly Australia, West Ryde, Australia) for 3 d. The medium was then changed to a medium comprised of high-glucose DMEM plus 10% FBS, 50 U/ml penicillin and 50  $\mu\text{g/ml}$  streptomycin and 0.166 U/ml insulin for 2 d. Cells were used only if more than 90% of the cells showed lipid droplet accumulation after differentiation. Total RNA was then extracted from the 3T3-L1 cells.

### ELISA

An ELISA was developed using commercially available unlabeled and biotinylated polyclonal antihuman chemerin antibodies (R&D Systems, Inc., Minneapolis, MN). Primary unlabeled antibody was diluted

to 1  $\mu\text{g}/\text{ml}$  in PBS and coated onto Maxisorp ELISA plates (Nunc, Chicago, IL) in 100  $\mu\text{l}$  at 4 C overnight. The plates were washed with PBS 0.05% Tween 20 (PBST) and blocked using 200  $\mu\text{l}$  blocking buffer (3% BSA in PBST) for 1 h. The blocking solution was removed, and plasma samples (diluted 1:60 with blocking buffer; 100  $\mu\text{l}$ /sample in duplicate) were added to the plate. After 2 h at room temperature, the plate was washed with PBST, and biotinylated antichemerin antibody (1  $\mu\text{g}/\text{ml}$ ) was added to each well. After 2 h the plate was washed, and streptavidin horseradish peroxidase (100 ng/ml; Sigma-Aldrich) was added to the plate. After 1 h at room temperature, the plate was washed with PBST, and the assay was developed using 100  $\mu\text{l}$ /well 3,3',5,5'-tetramethylbenzidine (0.1 mg/ml) dissolved in citrate buffer [50 mM  $\text{Na}_2\text{HPO}_4$  and 25 mM citric acid (pH 5.0)]. The reaction was stopped after 10 min by addition of 50  $\mu\text{l}$  1 M  $\text{H}_3\text{PO}_4$ . The assays were measured using a Bio-Rad microtiter plate reader (model 550; Bio-Rad Laboratories, Hercules, CA) at 450 nm with a reference of 630 nm. Interassay coefficient of variation was less than 10%, and the within-assay coefficient of variation was less than 5%. The sensitivity of the ELISA assay was 1–10 ng/ml, and the midrange of the assay was 5 ng/ml. The least detectable concentration of human chemerin was 0.5 ng/ml.

### Statistical analysis

Statistical analyses were performed using SPSS for Windows version 14.0 (SPSS, Inc., Chicago, IL). Differences between groups were compared using a one-way ANOVA. LeVeen's test for homogeneity of variance was used to determine whether variance between six animal groups (NGT fed, NGT fasted, IGT fed, IGT fasted, T2D fed, and T2D fasted) was equal or not. If homogeneity was not equal, the Games-Howell *post hoc* analysis was used, and if equal, least significant difference analysis was used to determine whether there were significant differences between groups or correlations with phenotypical characteristics. Differences and correlations were considered significant if  $P < 0.05$ .

Phenotypical parameters of human normal and type 2 diabetic subjects were compared using a Student's *t* test or a Mann-Whitney *U* test for data that were not normally distributed. Associations between circulating chemerin levels and phenotypical measures were determined using Pearson correlation (for normally distributed data) or Spearman correlation (for nonnormally distributed data) in SPSS. Multivariate

linear regression was used to determine whether associations were independent of other variables.

## Results

### Chemerin and CMKLR1 gene expression in tissues of *P. obesus*

A SST was developed to identify genes encoding secreted and membrane-bound proteins that were differentially expressed in tissues from NGT, IGT, or T2D *P. obesus*. Chemerin was identified from a SST performed using liver mRNA, however, chemerin was not differentially expressed in liver RNA samples of NGT or IGT or T2D *P. obesus* (data not shown). Subsequent gene expression analyses of chemerin and its receptor, CMKLR1, were performed in different tissues from *P. obesus*, and showed that both chemerin and CMKLR1 were expressed in all tissues tested, with highest expression of chemerin in liver, kidney, and adipose tissue (Fig. 1A). CMKLR1 was most highly expressed in adipose tissue and lung (Fig. 1B).

### Chemerin and CMKLR1 are differentially expressed in mesenteric fat of *P. obesus*

Because we had found that chemerin and CMKLR1 were highly expressed in adipose tissue, mRNA expression of these genes was explored in mesenteric adipose tissue of NGT, IGT, and T2D *P. obesus*. Chemerin gene expression was significantly higher in IGT and T2D *P. obesus* compared with the NGT group in the fasted state ( $P = 0.004$  and  $P = 0.01$ , respectively; Fig. 2A). Gene expression of CMKLR1 in mesenteric adipose tissue was significantly higher in IGT and T2D compared with NGT animals in the fed state ( $P = 0.009$  and  $P = 0.05$ , respectively). Chemerin gene expression was positively correlated with body weight ( $r = 0.647$ ;  $P = 0.002$ ), fasting blood glucose ( $r = 0.553$ ;

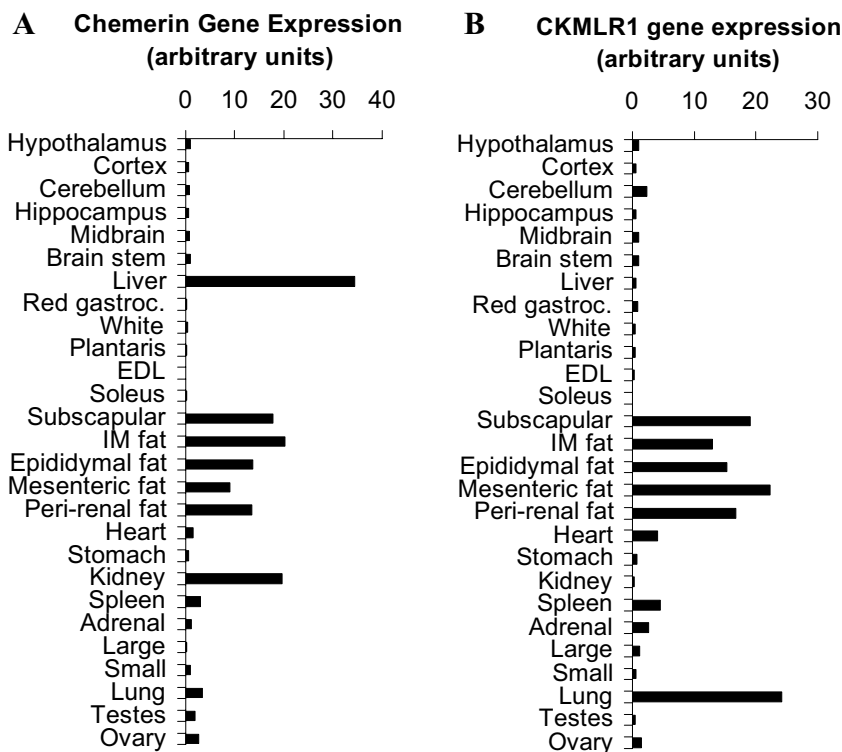


FIG. 1. Examination of chemerin and CMKLR1 expression in *P. obesus*. The relative mRNA expression of chemerin (A) and CMKLR1 (B) in various tissues of *P. obesus* was determined by real-time PCR. Data are represented relative to expression found in hypothalamus. EDL, Extensor digitorum longus; IM fat, im fat; Large, large intestine; Red gastroc, red gastrocnemius; Small, small intestine; White, white gastrocnemius.



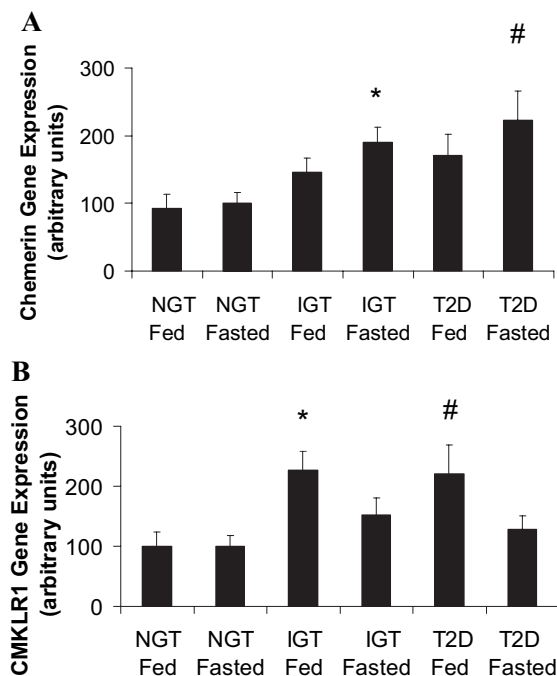


FIG. 2. Chemerin and CMKLR1 gene expression in mesenteric fat of *P. obesus*. The relative gene expression of chemerin and CMKLR1 in mesenteric adipose tissue from NGT, IGT, and T2D *P. obesus* was determined by real-time PCR. The data are represented as the mean  $\pm$  SE of each group. Each group comprises of six to eight animals, and animals in the fasted group were fasted for 24 h. Statistical analyses were performed using ANOVA. A, Chemerin gene expression was significantly higher in IGT and T2D animals compared with NGT animals in the fasted state (\*,  $P = 0.004$  and #,  $P = 0.01$ , respectively). B, CMKLR1 gene expression was significantly higher in IGT and T2D animals compared with NGT in the fed state (\*,  $P = 0.009$  and #,  $P = 0.05$ , respectively).

$P = 0.009$ ), and fasting plasma insulin ( $r = 0.872$ ;  $P < 0.001$ ). CMKLR1 expression was associated with body weight ( $r = 0.449$ ;  $P = 0.032$ ) and fasting plasma insulin levels ( $r = 0.524$ ;  $P = 0.012$ ). These observations show that chemerin gene expression is elevated in obese animals and is associated with characteristics of metabolic syndrome.

#### Gene expression of chemerin and CMKLR1 in sc and visceral adipose tissue

Cross-sectional studies in humans suggest that levels of visceral adipose tissue are more strongly associated with insulin resistance than levels of sc tissue (15). Therefore, it was of interest to examine gene expression levels of chemerin and CMKLR1 in both sc (subscapular) and visceral (mesenteric) adipose tissue from NGT, IGT, and T2D *P. obesus*. In sc adipose tissue, chemerin gene expression was significantly higher in both IGT and T2D compared with NGT animals ( $P = 0.006$  and  $P = 0.0001$ , respectively; Fig. 3A), whereas in visceral adipose tissue, chemerin was significantly higher in the T2D group compared with the NGT group ( $P = 0.01$ ). Gene expression of chemerin was significantly higher in visceral compared with sc adipose tissue in NGT animals ( $P = 0.03$ ; Fig. 3A). CMKLR1 gene expression was significantly higher in IGT compared with NGT sc adipose tissue ( $P = 0.03$ ) and showed a trend toward

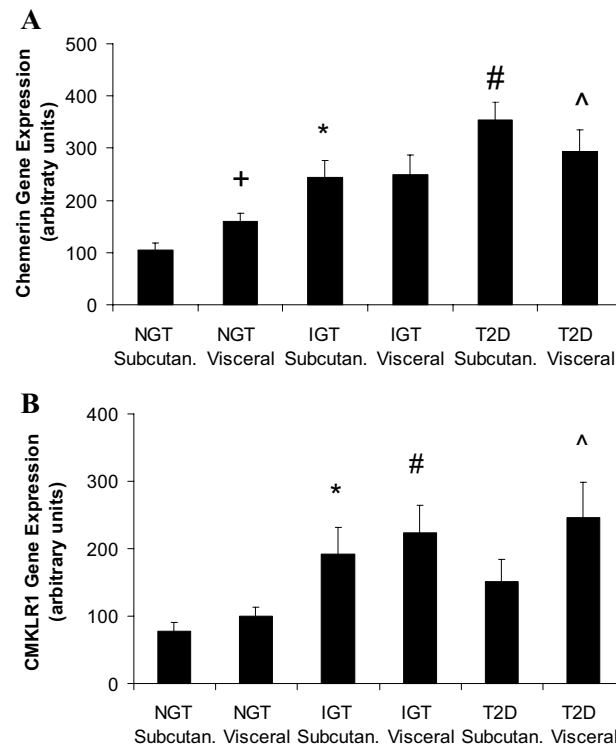


FIG. 3. Gene expression of chemerin and CMKLR1 in sc (Subcutan) and visceral fat of *P. obesus*. The relative gene expression of chemerin and CMKLR1 in the visceral and sc fat of NGT, IGT, and T2D *P. obesus* was determined by real-time PCR. The data are represented as the mean  $\pm$  SE of each group. Each group comprises of six to eight animals. A, Gene expression of chemerin was significantly higher in sc adipose tissue of IGT and T2D animals compared with the sc samples in the NGT group (\*,  $P = 0.006$  and #,  $P = 0.0001$ , respectively). Chemerin gene expression in visceral adipose tissue was significantly different in T2D animals compared with NGT animals (^,  $P = 0.01$ ). Chemerin was significantly higher in visceral compared with sc adipose tissue in NGT animals (+,  $P = 0.03$ ). B, CMKLR1 expression was higher in visceral adipose tissue in IGT and T2D animals compared with NGT animals (#,  $P = 0.02$  and ^,  $P = 0.03$ , respectively). CMKLR1 gene expression was significantly higher in sc adipose tissue from IGT animals compared with NGT animals (\*,  $P = 0.03$ ).

being higher in T2D animals, however, this difference was not statistically significant ( $P = 0.07$ ; Fig. 3B). Expression of CMKLR1 in visceral adipose tissue was higher in IGT ( $P = 0.02$ ) and T2D ( $P = 0.03$ ) compared with expression in visceral adipose tissue of NGT animals. In T2D animals, CMKLR1 gene expression was significantly higher in visceral compared with sc adipose tissue ( $P = 0.03$ ). However, CMKLR1 gene expression was not significantly different in sc compared with visceral adipose tissue in IGT or T2D animals ( $P = 0.57$  and  $P = 0.16$ , respectively). These results show that gene expression of chemerin and CMKLR1 are elevated in both sc and visceral adipose tissue in obese *P. obesus*.

#### Chemerin is predominantly expressed by adipocytes in adipose tissue

To determine which cells within adipose tissue express chemerin and CMKLR1, mesenteric adipose tissue from NGT and IGT *P. obesus* was fractionated into cellular populations comprised of adipocytes and stromal-vascular cells.

Chemerin expression was significantly higher in adipocytes compared with stromal-vascular cells in both NGT and IGT animals ( $P = 0.0001$ ), and chemerin expression was significantly higher in adipocytes of IGT compared with the adipocytes of NGT animals ( $P = 0.01$ ; Fig. 4). In contrast, CMKLR1 gene expression was not significantly different in adipocyte and stromal-vascular cells from adipose tissue of *P. obesus* (data not shown). These results show that in adipose tissue, chemerin is predominantly expressed within adipocytes, whereas CMKLR1 is expressed by both adipocytes and stromal-vascular cells.

#### Effects of 3T3-L1 adipocyte differentiation on chemerin and CMKLR1 expression

*In vitro* experiments in 3T3-L1 adipocytes were used to determine chemerin and CMKLR1 gene expression during differentiation of fibroblasts into mature adipocytes. Chemerin gene expression was markedly up-regulated during differentiation (d 3, 4, 5, and 6;  $P < 0.001$ ) and was approximately 20-fold higher in fully differentiated adipocytes compared with undifferentiated fibroblasts (d 7, 8, and 9;  $P < 0.001$ ; Fig. 5A). In contrast, CMKLR1 gene expression was down-regulated during differentiation (d 1, 2, and 3;  $P < 0.001$ ) compared with d 0 and was approximately 10-fold lower in fully differentiated adipocytes (d 7, 8, and 9;  $P < 0.001$ ; Fig. 5B). These results confirm that the chemerin expression observed in adipose tissue was predominantly expressed within adipocytes, however, mature adipocytes express reduced levels of CMKLR1 compared with fibroblasts.

#### Serum chemerin levels are correlated with metabolic syndrome-related phenotypes

To determine whether circulating levels of chemerin were associated with T2D and/or measures of obesity, plasma levels of chemerin were measured by ELISA in human plasma samples from 256 individuals aged 35–65 yr, of whom 142 were NGT, and 114 had T2D. The phenotypical characteristics of each group are presented in Table 1. Plasma chemerin levels were not significantly different between the NGT and T2D groups.

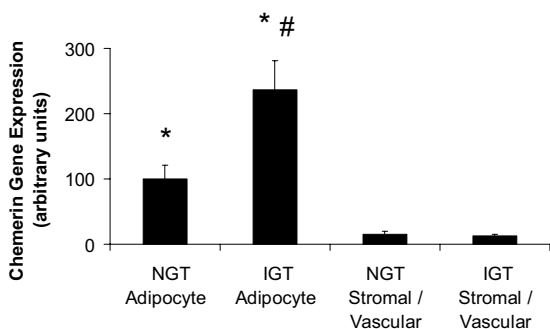


FIG. 4. The relative gene expression of chemerin in fractionated mesenteric adipose tissue. Chemerin gene expression in adipocytes and stromal and vascular cells isolated from mesenteric adipose tissue from *P. obesus* was determined by real-time PCR and is represented as the mean  $\pm$  SE of each group comprised of six animals. Chemerin gene expression was significantly higher in adipocytes compared with stromal-vascular cells in both NGT and IGT animals (\*,  $P = 0.0001$ ), and chemerin expression was significant higher in adipocytes of IGT compared with the adipocytes of NGT animals (#,  $P = 0.01$ ).

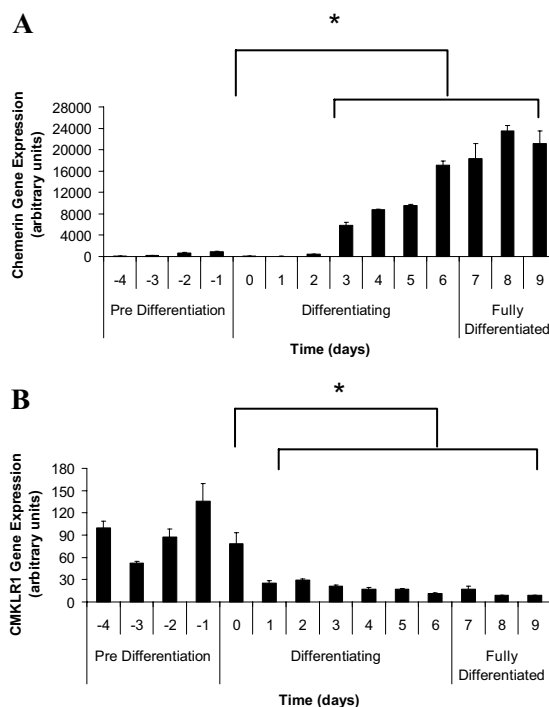


FIG. 5. Chemerin and CMKLR1 gene expression in differentiating 3T3-L1 adipocytes. Relative gene expression of chemerin and CMKLR1 was determined for each day during the differentiation of 3T3-L1 cells from fibroblasts into mature adipocytes. Data are expressed as the mean  $\pm$  SE, with each treatment performed in triplicate. A, Chemerin expression was significantly up-regulated after 3-d differentiation (\*,  $P < 0.001$ ) compared with d 0. B, CMKLR1 expression was significantly down-regulated after 3-d differentiation (\*,  $P < 0.001$ ) compared with d 0.

We also tested whether there was a difference in plasma chemerin levels between NGT subjects with BMI more than 30 kg/m<sup>2</sup> ( $n = 18$ ) and those with less than 25 kg/m<sup>2</sup> ( $n = 75$ ). Plasma chemerin levels were significantly higher in subjects with BMI more than 30 kg/m<sup>2</sup> compared with those with less than 25 kg/m<sup>2</sup> ( $296.5 \pm 61.2$  vs.  $222.7 \pm 67.1$  ng/ml,  $P = 6.2 \times 10^{-5}$ ). Linear regression analysis was performed to determine whether plasma chemerin levels were associated with adiposity and/or metabolic syndrome related phenotypes. Strong correlations with age and gender were observed due to higher chemerin levels in females compared with males and older individuals compared with younger individuals. After adjusting for age and gender, chemerin levels were significantly associated with measures of body fat [BMI, fat mass, weight, and waist hip ratio (WHR)] and metabolic syndrome-related phenotypes (fasting glucose, fasting insulin, plasma triglycerides, and blood pressure; Table 2) in NGT subjects. After further adjustment for BMI, plasma chemerin levels were still independently associated with metabolic syndrome-related phenotypes, including systolic blood pressure ( $P = 0.001$ ) and plasma triglycerides ( $P = 0.009$ ), but not measures of insulin sensitivity or glucose homeostasis. When the data were adjusted for age, gender, BMI, and triglycerides, chemerin levels were only associated with systolic and diastolic blood pressure ( $P = 0.002$  and  $P = 0.004$ , respectively). These results clearly demonstrate that circulating chemerin levels are associated with key char-

TABLE 1. Physical and metabolic characteristics of study subjects

Phenotype	NGT (n = 142)		T2D (n = 114)		Significance (P value)
	Mean ± SD	Range	Mean ± SD	Range	
Age (yr)	47 ± 11	30–77	51 ± 10	27–79	0.001
Fasting glucose (mmol/liter)	5.2 ± 0.4	4.0–6.0	8 ± 3	5–17	<0.0001
Two-hour glucose (mmol/liter)	6 ± 1	1–8	15 ± 5	5–30	<0.0001
Fasting serum insulin (U/ml)	10 ± 6	1–38	14 ± 9	2–60	<0.0001
Two-hour serum insulin (U/ml)	64 ± 55	2–428	76 ± 63	5–412	0.062
Homa_b	120 ± 46	33–274	77 ± 44	8–201	<0.0001
Homa_s	53 ± 34	11–264	35 ± 29	6–230	<0.0001
Height (cm)	161 ± 9	140–183	159 ± 9	138–180	0.056
Weight (kg)	66 ± 12	41–111	68 ± 13	38–120	0.091
BMI (kg/m <sup>2</sup> )	25 ± 4	17–44	27 ± 5	16–42	0.002
WHR	0.9 ± 0.1	0.7–1.0	0.9 ± 0.1	0.7–1.2	<0.0001
Fat mass (kg)	21 ± 7	3–41	22 ± 7	6–42	0.03
Body fat (%)	31 ± 9	6–50	33 ± 8	15–56	0.057
Total cholesterol (mmol/liter)	5 ± 1	3–8	5.3 ± 1.6	2–15	0.016
HDL (mmol/liter)	0.9 ± 0.3	0.2–1.9	0.8 ± 0.3	0.2–2.0	0.054
TG (mmol/liter)	1.4 ± 0.7	0.4–5.3	2.0 ± 1.7	0.6–15.8	<0.0001
Systolic BP	124 ± 20	94–199	137 ± 21	100–213	<0.0001
Diastolic BP	75 ± 13	47–127	80 ± 13	47–141	0.0002
Chemerin	249 ± 71	114–437	250 ± 67	86–530	0.87

BP, Blood pressure; HDL, high-density lipoprotein; Homa\_b, quantitative estimate of b-cell function; Homa\_s, quantitative estimate of insulin sensitivity; TG, triglycerides.

acteristics of metabolic syndrome, including BMI, plasma tri-  
glyceride levels, and blood pressure.

Discussion

Secreted proteins are central regulators of metabolism, and play  
key roles in food intake, insulin sensitivity, and energy metabo-  
lism. We have developed a SST to identify novel genes that encode  
secreted or membrane-bound proteins in metabolically important  
tissues, such as liver, muscle, and adipose tissue. Using this ap-  
proach, we detected the chemokine chemerin, and subsequently  
demonstrated that chemerin and its receptor, CMKLR1, were  
highly expressed in adipose tissue. In addition, we have shown  
that expression of both chemerin and CMKLR1 were up-regulated  
in adipose tissue of obese *P. obesus*. Circulating levels of chemerin  
were significantly associated with characteristics of metabolic syn-

drome (circulating triglycerides, blood pressure, body fat content,  
and insulin resistance) in NGT human subjects. These studies, for  
the first time, identify chemerin as a novel adipokine, which may  
play a role in the pathophysiology of obesity and metabolic  
syndrome.

Chemerin has been expressed in a number of tissues, includ-  
ing liver, pancreas, and lung (16–18), whereas expression of  
CMKLR1 has previously been found predominantly in cells of  
the immune system, such as neutrophils, activated macro-  
phages, and dendritic cells (17). Here we extend the range of  
tissues known to express chemerin and CMKLR1 to include  
adipose tissue. By fractionating adipose tissue into cellular com-  
ponents, we have also shown that chemerin is predominantly  
expressed by mature adipocytes within adipose tissue. This  
finding is consistent with the marked increase in chemerin

TABLE 2. Chemerin is associated with metabolic syndrome-related phenotypes

Phenotype	Adjusted for age and gender	Adjusted for age, gender, and BMI	Adjusted for age, gender, BMI, and TG	Adjusted for age, gender, BMI, TG, and systolic BP
BMI (kg/m <sup>2</sup> )	$P = 6.3 \times 10^{-05}, r = 0.324$			
TGs (mmol/liter)	$P = 0.002, r = 0.264$	$P = 0.009, r = 0.219$		
Systolic BP	$P = 4.8 \times 10^{-05}, r = 0.334$	$P = 0.001, r = 0.275$	$P = 0.002, r = 0.265$	
Diastolic BP	$P = 0.0002, r = 0.306$	$P = 0.004, r = 0.240$	$P = 0.004, r = 0.241$	$P = 0.668, r = 0.036$
Fat mass (kg)	$P = 0.003, r = 0.252$	$P = 0.924, r = 0.008$	$P = 0.963, r = 0.004$	$P = 0.920, r = 0.002$
Weight (kg)	$P = 0.0002, r = 0.306$	$P = 0.847, r = -0.016$	$P = 0.662, r = -0.037$	$P = 0.615, r = -0.043$
WHR	$P = 0.007, r = 0.255$	$P = 0.244, r = 0.098$	$P = 0.389, r = 0.073$	$P = 0.788, r = 0.023$
Body fat (%)	$P = 0.07, r = 0.152$	$P = 0.650, r = 0.038$	$P = 0.663, r = 0.037$	$P = 0.618, r = 0.042$
Homa_s	$P = 0.0004, r = -0.242$	$P = 0.195, r = -0.109$	$P = 0.533, r = -0.053$	$P = 0.993, r = -0.001$
Homa_b	$P = 0.104, r = 0.137$	$P = 0.99, r = -0.001$	$P = 0.528, r = -0.054$	$P = 0.0348, r = -0.08$
Fasting insulin (U/ml)	$P = 0.001, r = 0.287$	$P = 0.215, r = 0.105$	$P = 0.569, r = 0.048$	$P = 0.943, r = -0.006$
Two-hour insulin (U/ml)	$P = 0.041, r = 0.173$	$P = 0.655, r = 0.038$	$P = 0.864, r = -0.015$	$P = 0.581, r = -0.047$
Fasting glucose (mmol/liter)	$P = 0.032, r = 0.180$	$P = 0.077, r = 0.149$	$P = 0.088, r = 0.144$	$P = 0.122, r = 0.131$
Two-hour glucose (mmol/liter)	$P = 0.032, r = 0.180$	$P = 0.043, r = 0.170$	$P = 0.08, r = 0.148$	$P = 0.241, r = 0.099$
Total cholesterol (mmol/liter)	$P = 0.05, r = 0.165$	$P = 0.121, r = 0.131$	$P = 0.717, r = 0.116$	$P = 0.993, r = 0.084$
Height	$P = 0.977, r = 0.002$	$P = 0.739, r = -0.028$	$P = 0.555, r = -0.05$	$P = 0.52, r = -0.055$
HDL	$P = 0.01, r = -0.165$	$P = 0.618, r = -0.042$	$P = 0.420, r = 0.068$	$P = 0.323, r = 0.084$

Associations were performed using multivariate linear regression. The data were adjusted for age, gender, BMI, and plasma triglycerides (TGs). BP, Blood pressure; HDL, high-density lipoprotein; Homa\_b, quantitative estimate of b-cell function; Homa\_s, quantitative estimate of insulin sensitivity.

<sup>a</sup> Values were obtained using Spearman's correlation; all other data were obtained using Pearson's correlations.

expression during differentiation of 3T3-L1 adipocytes *in vitro*. These observations demonstrate that chemerin is expressed in mature but not preadipocytes. In contrast to chemerin, CMKLR1 was expressed in both adipocytes and stromal-vascular cells, which suggests that both components of adipose tissue may be responsive to chemerin signaling.

The metabolic syndrome is a cluster of metabolic and cardiovascular disturbances, such as central obesity, hypertension, dyslipidemia, and hyperglycemia (19). The molecular basis of metabolic syndrome has not been fully elucidated (20), but obesity plays a central role. Adipose tissue produces and secretes adipokines, and their dysregulation in visceral obesity, may play a role in the development of metabolic syndrome (21). For example, proinflammatory cytokines, including TNF- $\alpha$ , activate nuclear transcription factor- $\kappa$ B and induce oxidative stress, which lead to dyslipidemia, glucose intolerance, insulin resistance, hypertension, endothelial dysfunction, and atherogenesis (22). Plasma levels of chemerin showed a strong and independent association with key markers of the metabolic syndrome, including obesity, plasma triglycerides, and blood pressure. This finding suggests that chemerin may play a role in the development of these metabolic syndrome phenotypes. Moreover, it raises the possibility that chemerin may be of value as a biomarker for this disorder.

Plasma levels of chemerin were found to be strongly associated with blood pressure in NGT subjects, which suggests that chemerin may also be a novel regulator of blood pressure. Chemerin is structurally related to other circulating factors, including cathelicidins, cystatins, and kininogen (17). It is notable that a proteolytic product of kininogen is the vasoactive peptide bradykinin. In addition, it is also of interest that chemerin is highly expressed within the kidney, a key site of blood pressure regulation. Together, these observations suggest that further investigation of the role of chemerin in the regulation of blood pressure is warranted.

The source of chemerin protein found in the circulation is unclear at present. The chemerin gene encodes a signal peptide and is secreted from several cells types, such as endothelial cells (23) and 3T3-L1 fibroblasts (data not shown). Chemerin mRNA was found to be most highly expressed in liver, adipose tissue, and kidney. Although the presence of chemerin mRNA in a cell does not guarantee synthesis or secretion of the encoded protein, it is likely that these tissues contribute significantly to plasma chemerin levels. Because chemerin mRNA expression was found to be increased in adipose tissue but not liver from obese compared with lean *P. obesus*, it is tempting to speculate that elevated chemerin levels found in the plasma of obese humans may originate from adipose tissue. Future work is clearly required before the source of plasma chemerin can be conclusively determined.

Recent studies (7, 8) have shown that obesity induces inflammation in adipose tissue. Because chemerin is a proinflammatory cytokine that recruits and activates immune cells, it is possible that chemerin may play a role in the inflammation of adipose tissue that occurs in obesity. In this study we have examined chemerin expression in adipose tissue in a cross-section of young adult *P. obesus*, so it is not possible to conclude a causative role for chemerin in obesity associated inflammation of adipose tissue from these studies. Mapping chemerin expression during diet-induced obesity or using chemerin neu-

tralizing antibodies in animal models of diet-induced obesity may help resolve this issue.

We have found that chemerin mRNA was highly expressed in mature adipocytes and was increased in adipose tissue of obese animals. These findings are similar to the expression of the adipokine leptin in adipose tissue (24, 25). These observations suggest that chemerin expression may reflect the state of differentiation of adipocytes, adipocyte cell size, or total body fat mass. Chemerin mRNA was markedly up-regulated during differentiation of 3T3-L1 cells, whereas expression of CMKLR1 mRNA was down-regulated, which raises the possibility that chemerin may negatively feedback on the expression of CMKLR1. This mode of regulation by a chemokine on the expression of its receptor is commonly observed for many chemokines and dampens otherwise potentially dangerous overactivation of chemokine-regulated processes such as production of toxic molecules (*e.g.* reactive oxygen intermediates, nitric oxide) or cell proliferation (26). However, we have found that exposure of 3T3-L1 preadipocytes to active recombinant murine chemerin for 24 h did not affect CMKLR1 mRNA expression (data not shown). In addition, CMKLR1 mRNA expression was markedly decreased at 24 h after the start of differentiation, whereas chemerin expression was markedly increased only after 72-h differentiation. Together, these findings suggest that, at least in preadipocytes, chemerin may not regulate CMKLR1 expression. In contrast to the reciprocal expression of chemerin and CMKLR1 mRNA during the differentiation of 3T3-L1 cells, the expression of both of these genes in adipose tissue was increased in IGT and T2D *P. obesus*. Because adipogenesis is increased in these animals, these data are also consistent with chemerin playing a limited role in the regulation of CMKLR1 expression in adipocytes and adipose tissue.

Chemerin protein exists as a full-length protein and a short form that is produced by removal of 5–10 amino acids at the C-terminal end of the chemerin protein by serine proteases, such as neutrophil elastase, cathepsin G, plasmin and C1s (18, 27, 28). The full-length isoform of chemerin has significantly lower bioactivity compared with the proteolytically processed short form. Circulating chemerin in plasma exists primarily as the full-length isoform and is converted to the bioactive short isoform at sites where serine proteases are expressed, such as at a site of inflammation or clotting of blood (27, 29). It is unclear which form of chemerin is found in adipose tissue, however, several known chemerin-activating proteases such as C1s (30, 31) and cathepsin G (32) are expressed in adipose tissue. These observations lead to the hypothesis that chemerin in adipose tissue from obese animals may be proteolytically cleaved to the bioactive form, whereas in lean animals chemerin remains as the full-length, inactive form. Therefore, this potential regulation of chemerin bioactivity may be a key early initiator of downstream processes, such as adipogenesis or inflammation. Moreover, it will be of interest to determine whether adipocyte serine proteases such as adipsin are proteolytically active toward chemerin or if adipocyte serine protease inhibitors such as plasminogen activator inhibitor 1 and vaspain prevent chemerin processing.

Previous reports have shown that expression of chemerin and CMKLR1 may be induced by retinoic acid (16, 33). Notably, recent studies show that RBP4 is highly expressed in adipose tissue, and this expression increases with obesity (10). Because



RBP4 is the major circulating transporter of retinoic acid (9), elevated RBP4 levels associated with obesity may lead to increased delivery of retinoic acid to adipose tissue of obese animals, and subsequent up-regulation of chemerin and CMKLR1 expression. Therefore, it will be of interest to determine chemerin expression in RBP4 deficient animals or mice fed a retinoic acid-deficient diet to resolve the role of retinoic acid in chemerin and CMKLR1 expression in adipose tissue.

In summary, we have for the first time demonstrated that chemerin is an adipokine that exhibits increased mRNA expression in adipose tissue of obese animals. Subsequent characterization of plasma chemerin levels in humans clearly demonstrated a relationship between this novel adipokine and several key aspects of metabolic syndrome.

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