Chemerin is expressed mainly in pancreas and liver, is regulated by energy deprivation and lacks day/night variation in humans

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Abstract

Objective—Chemerin is an adipocyte-secreted hormone, recently associated with obesity and the metabolic syndrome. Although studies in rodents have outlined aspects of chemerin's function and expression, its physiology and expression patterns are still to be elucidated in humans.

Methods—To evaluate for any day/night variation in chemerin secretion we analyzed hourly serum samples from six females in the fed state. To examine whether energy deprivation affects chemerin levels, and whether this could be mediated through leptin, we analyzed samples from the same subjects in the fasting state while we were administering either placebo or leptin. To evaluate for any potential dose-effect relationship between leptin and chemerin, we administered increasing metreleptin doses to five females. A tissue array was utilized to study the expression of chemerin in different human tissues. Ex vivo treatment of human fat explants from 3 subjects with leptin was performed to evaluate for any direct effect of leptin on adipocyte chemerin secretion.

Results—Chemerin does not display a day/night variation, while acute energy deprivation resulted in a significant drop in circulating chemerin levels by ~42%. The latter was unaltered by metreleptin administration and leptin administration did not affect secretion of chemerin by human adipose tissue studied *ex vivo*. Chemerin was expressed primarily in the adrenal gland and liver. Chemerin receptor showed increased expression in lymph nodes and the spleen.

Conclusions—We outline for the first time chemerin expression and physiology in humans which is different from mice.

Keywords

Chemerin; Day/Night Variation; Energy Homeostasis; Leptin; sTNFRII; ex vivo; tissue; mRNA expression

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Introduction

With the emergence of obesity as a major health hazard, several adipose tissue secreted proteins and hormones, known as adipokines, have been identified and linked to metabolic regulation. Changes in the secretion of many of these molecules are now thought to play a major role in the development of obesity and obesity related diseases. Chemerin is one such protein that has recently been proposed to be an adipokine. It is secreted as an inactive 18-kDa pro-peptide and is subsequently cleaved to a 16-kDa active protein that binds to the G protein-couple receptor, chemokine-like receptor 1 (CMKLR1) [1]. Chemerin was first identified in psoriatic skin lesions [2] and is now known to be involved in the recruitment of immune cells such as immature dendritic cells, macrophages, natural killer cells, and monocytes to sites of tissue inflammation or injury [3]. Chemerin is associated with proinflammatory cytokines such as TNF- both *in vitro* and *in vivo* [4, 5]. Recently it was demonstrated that chemerin promotes angiogenesis in endothelial cells [6, 7]. A major role of chemerin is adipogenesis regulation and it has also been associated with the metabolic syndrome [8–10]. Chemerin has been shown to be correlated with body mass index (BMI), homeostasis model assessment of insulin resistance (HOMA-IR), blood pressure, and circulating triglycerides [10, 11] in humans. Furthermore, increased chemerin correlates with other obesity related markers such as leptin, resistin, and C - reactive protein (CRP) as well as glycated hemoglobin (HbA1C) [12–14]. *In vitro* experiments have demonstrated that chemerin is an important regulator of adipocyte differentiation and metabolism, as exhibited by the knockdown of chemerin causing an impaired pre-adipocyte differentiation into mature adipocytes as well as reduced expression of glucose and lipid metabolism genes[8, 15]. However, little is known about the circulatory or expression patterns of chemerin in humans.

Although chemerin has been proposed to be an adipokine, the expression pattern of chemerin in human tissues has not yet been evaluated comparatively. Furthermore, the physiology of chemerin in humans remains to be fully elucidated. No studies to date have determined whether chemerin displays a day/night pattern of secretion and what the effect of acute and/or chronic energy deprivation on circulating chemerin levels in humans is. It is expected that circulating levels of chemerin, a factor associated with energy homeostasis, would respond appropriately to changes in energy intake. Finally, any potential interactions between chemerin and other molecules affected by energy intake such as leptin have not yet been studied. Leptin is a 167 amino acid adipokine [16] that has a well-established role in the neuroendocrine regulation of energy homeostasis [17, 18]. Recent studies have demonstrated an association between leptin levels, obesity, and the metabolic syndrome [17–19]. During states of starvation circulating levels decrease and are responsible for changes in the neuroendocrine axis [20]. In mouse models, leptin has been shown to act on the hypothalamic pro-opiomelanocortin neurons (POMC) to decrease food intake and maintain glucose homeostasis [21]. Studies in patients with conditions characterized by chronic energy deprivation, such as anorexia nervosa and hypothalamic amenorrhea, have demonstrated that low leptin levels are causally related to energy deprivation induced suppression of neuroendocrine axes whereas leptin administration restores these energy deprivation induced changes [20, 22–24].

We utilized herein a human mRNA tissue array to evaluate the expression pattern of chemerin and its receptor in human tissues. We then proceeded to assess whether chemerin has a day/night variation pattern in humans, to determine whether acute energy deprivation (for 3 days) alters chemerin levels, to investigate for potential association with changes in circulating TNF- as measured by TNFRII as well as leptin mediated effects of energy deprivation in humans, and to examine the tissue expression of chemerin. To these ends, we first utilized frequently collected samples from six healthy female individuals studied while in the isocaloric fed state, after 3 days of fasting and 3 days of fasting plus metreleptin

administration; then we used samples collected from another five healthy female subjects while treated with low, physiological, and supraphysiological doses of leptin; and finally, we performed ex vivo experiments involving adipose tissue surgically removed from human subjects.

Methods

Chemerin and CMKLR1 Tissue mRNA Expression

Chemerin and CMKLR1 mRNA expression was measured using human-specific TaqMan® Gene Expression Assays (Assay ID: Chemerin, Hs00161209_g1; CMKLR1, Hs01081979_s1, Applied Biosystems, Foster City, CA) in 7500 Fast Real-Time PCR system using Standard real-time 7500 protocol. Data were analyzed using 7500 system software (Applied Biosystems, Foster City, CA) and relative quantification was done using Ct method with beta-actin as the internal control. Analysis of tissue mRNA expression was done in duplicate with TissueScan™ Real-Time Human Major Tissue Panel from OriGene, Rockville, MD, containing 48 tissue types.

Study 1

Day/Night variation analysis and effect of acute energy deprivation—To evaluate whether chemerin has a day/night variation pattern we collected serum samples from six healthy, lean, eumenorrheic women (age 22.8 ± 3.4 years; BMI 21.7 ± 2.2 kg/m^2) initially studied in the isocaloric fed state [25]. All female subjects had a regular menstrual cycle and were not taking any medications including oral contraceptive pills. Study visits were standardized at the 6th to 11th day of their menstrual cycle. The study was approved by the Institutional Review Board at the Beth Israel Deaconess Medical Center (BIDMC) and all participants gave informed consent. In order to evaluate whether energy deprivation has any effect on chemerin levels, and whether such a change could be mediated through leptin, we collected samples from the same subjects on two separate admissions, spaced at least 8 weeks apart, in which subjects were randomized to receive either placebo or leptin during complete fasting for three days. Admissions in each state were held in the General Clinical Research Center (GCRC) of BIDMC for 4 days with standardized diet, exercise, and light/ dark intervals. Baseline blood samples were taken at 8 a.m. day 1 of each visit before intervention commenced. On day 3 of all 3 visits blood samples were drawn every hour starting at 8 a.m. for 24 hours. For the admission in the fed state subjects were given an isocaloric diet with breakfast at 8 a.m., lunch at 1 p.m., dinner at 6 p.m., and a snack at 10 p.m. The caloric intake was distributed so that 20% of calories were from breakfast, 35% from lunch, 35% from dinner, and 10% from the evening snack. For admissions in the fasting state treatments with placebo or metreleptin were performed in a double-blinded, randomized, cross-over fashion. Replacement doses of metreleptin were given as 4 subcutaneous injections per day every 6 hours starting at 8 a.m. on day 1. Metreleptin was administered at doses of 0.08 mg/kg/day on day 1 and 0.2 mg/kg/day on days 2 and 3. Placebo treatment was carried out with the same schedule as well as the same volume and mode of administration as during leptin treatment.

Study 2

Effect of increasing Doses of Leptin on Chemerin Circulation—To evaluate for any potential dose-effect relationship of metreleptin administration on chemerin levels, we evaluated five healthy female volunteers in a cross-over study of administration of low (0.01 mg/kg), physiological (0.1 mg/kg), and high (0.3 mg/kg) metreleptin doses on three separate clinical visits[26]. The mean age and BMI of the subjects was 20.4 ± 0.7 years and 21.9 ± 0.7 kg/m2 accordingly. All female subjects were in the isocaloric fed state had a regular menstrual cycle and were not taking any medications including oral contraceptive pills. The

study was approved by the Institutional Review Board at the Beth Israel Deaconess Medical Center (BIDMC) and all participants gave informed consent. One day admissions in each state were held in the General Clinical Research Center (GCRC) of BIDMC with samples taken at 8 a.m., 12, 4, and 8 p.m.. Leptin at all doses was administered once at 8 a.m.

Ex Vivo Treatment of Adipose Tissue

Ex vivo procedures were carried out as previously reported [27]. In short, human subcutaneous and omental fat explants from 3 subjects (2 Female, 1 male, age 51.7 ± 9.5 years, BMI 43.8 ± 5.8 kg/m^2) were placed into Krebs-Ringer-HEPES buffer (20 mmol/l, pH 7.4) containing 2.5% BSA and 200 nmol/l adenosine in the operating room and immediately taken to the laboratory. Tissue was then minced and all nonadipose tissue was removed by rinsing with fresh buffer. The samples were divided equally and incubated in 37°C shaking water bath with or without leptin treatment (100 ng/mL) for 20 hours. Chemerin secretion was measured via ELISA of the supernatant. Subcutaneous adipose tissue protein levels were measured by western blot analysis.

Western Blotting

Western blotting was done as previously described [27]. In short, the proteins were loaded in each lane and underwent SDS-polyacrylamide gel electrophoresis. Proteins were then blotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The membranes were blocked for 20 minutes in TBS containing 5 % nonfat dry milk and 0.1 % Tween-20. Primary antibodies were incubated in TBS containing 5 % nonfat dry milk overnight and then incubated with horseradish peroxidase secondary antibodies for 2 hours. After incubation with all antibodies membranes were washed with TBS containing 0.1 % Tween-20. Enhanced chemiluminescence was used for detection. Measurement of signal intensity on nitrocellulose membranes was performed using Image J processing and analysis software.

Hormone Measurements

Serum chemerin was measured by ELISA (Biovendor, Chandler, NC, USA) with a sensitivity of 0.1 ng/mL and intra-assay CV of 5.1 – 7.0% and inter-assay CV of 6.9 – 8.3% [28, 29]. Serum sTNFRII was measured by ELISA (R&D Systems, Minneapolis, MN, USA) with a sensitivity of 0.6 pg/mL, intra-assay CV of 2.6 – 4.8% and inter-assay CV of 3.5 – 5.1%. Serum Leptin, Insulin, IGF-1, and FFA were measured as previously reported[25].

Statistical Analysis

Statistical analysis was performed with STATA v11.2 (STATA Corp. College Station, TX) and Pulse XP (University of Virginia, Charlottesville, VA) software. Results are presented as means ± standard deviation. Normality of the dependent variables was evaluated with the Shapiro-Wilkes test of normality and P-P plots. Evaluation of the existence of a day/night variation pattern for chemerin was performed by hour to hour comparison across a 24 hour period using repeated measurements analysis of variance. Possible dominant harmonic components were analyzed on a subject to subject basis by spectral domain analysis for confirmation, using stata *pergram* routine. Chemerin, leptin, and sTNFRII levels were also analyzed for potential day night variation, by 4 parameter, cosine, nonlinear regression models, evaluating the adjusted coefficient of determination (R^2). Comparisons of all molecules levels between the three states (fed, fasting + placebo and fasting + leptin) were performed with hierarchical mixed effects linear modeling. Chemerin levels were modeled as a linear function of time, at the level of each individual. Fixed effects of each state on the intercept and slope of the growth trajectories were assessed by introducing "state" as a level-2 dummy variable. Comparisons between day 1 and day 4 between and within each

group as well as the study 2 leptin-dose response were performed by repeated measures ANOVA. Correlations between chemerin and insulin levels in the fasting state were evaluated with simple linear regression. Analysis of chemerin levels in *ex vivo* experiment was done by paired T-Test. All p-values are two sided, alpha criterion was set to 0.05.

Results

Tissue mRNA Expression of Chemerin and CMKLR1

Values of chemerin and CMKLR1 mRNA expression levels are displayed as relative to adrenal gland levels. Chemerin levels are distributed throughout multiple tissues with the highest expression seen in pancreas, liver, adrenal gland, kidney (Fig. 1), and rectum (data not shown). Chemerin is expressed the least in the thymus, brain, plasma leukocytes, and placenta. The expression of CMKLR1 is more widely distributed while the highest levels are seen in tissues important in immune or inflammatory responses such as the spleen, lymph node, and vena cava (Fig. 1). Other tissues analyzed for chemerin and CMKLR1 include muscle, brain, intracranial nerves, retina, optic nerve, urethra, bladder, vagina, penis, uvula, oviduct, seminal vesicles, prostate, cervix, mammary gland, pituitary, thyroid, pericardium, tonsils, esophagus, nasal mucosa, trachea, tongue, spinal cord, epididymis, and skin (data not shown).

Chemerin in Circulation

Baseline characteristics including weight, BMI, insulin, Free Fatty Acids (FFA), chemerin, Serum soluble tumor necrosis factor receptor II (sTNFRII), and leptin of the 6 subjects in all three conditions (fed, fasting plus placebo, and fasting plus metreleptin) are outlined in Table 1. Only the fasting plus metreleptin administration state demonstrated a slight decrease in body weight and BMI. Day 4 measurements of insulin, leptin, FFA, sTNFRII and chemerin were not statistically different from day 1 in the fed state. Insulin, leptin, and chemerin all decreased in response to fasting on day 4 compared to day 1. In contrast, FFA increased in response to fasting. Soluble TNFRII was unchanged in all 3 states. Leptin was increased on day 4 in the fasting with metreleptin administration state while all other hormones remained in ranges seen in the fasting with placebo administration condition (Table 1).

Day/Night Variation of Chemerin

Repeated measurements analysis of variance on normalized chemerin levels revealed no statistically significant difference between hourly time points (p=0.169). The nonlinear cosinor regression analysis of chemerin over the 24 hour period demonstrated a statistically significant day/night variation in both chemerin and sTNFRII circulating levels during the fed state (adjusted coefficients of determination: $R^2= 8.45\%$, p<0.01 and $R^2=4.8\%$, p=0.02, accordingly). Although statistically significant, we believe that the coefficient of determination calculated for chemerin and sTNFRII was of not clinically significant magnitude. There are no available guidelines on what level of coefficient of determination, derived from non-linear regression models, should serve as a threshold to characterize a model as "physiologically" or "clinically" significant and the statistical consensus recommends that this decision should be made on a study-to-study base, based on the pre-exiting literature. Cortisol and leptin are hormones with established day/night variability secretion patterns and thus using them as a reference is relevant. Previous studies from our group have demonstrated that cortisol, yields an adjusted coefficient of determination of more than 30% in similar statistical models[30], while in the present study leptin yielded an adjusted coefficient of determination of 27.66% (p<0.01). Given the fact that the coefficient of determination for hormones with well-established day/night variation patterns is in the 25-30% range we support the notion that the 4.8-8.45% level observed in chemerin and

sTNFRII circulating levels is not of clinical significance. Leptin levels displayed a significant day/night rhythm during the fed state with a peak during the night (Figure 2)

Effects of Acute Energy Deprivation on Chemerin

Circulating chemerin levels were significantly lower after a 72-hour fasting than during the fed state (p<0.001). Similarly, leptin displayed decreased circulating levels in the fasting state compared to the fed state (p<0.001). However, sTNFRII levels were not affected by a 72-hour fast (p=0.557) (Fig. 3).

Leptin Effects on Chemerin

Leptin levels showed a significant drop during the fasting state as compared to the fed state. Leptin replacement in the fasting state by metreleptin administration completely restored serum leptin levels to the physiological range (Table 1). On the contrary, chemerin levels were significantly lower in fasting state during metreleptin administration compared to during the fed state (p<0.001). Also, levels of chemerin during leptin replacement were not statistically different from levels during fasting plus placebo (p=0.84). Furthermore, levels of sTNFRII were unaffected by treatment with recombinant leptin (p=0.715) (Fig. 3).

Leptin treatment at high, physiological, and low doses showed no difference in chemerin circulating levels (Fig. 4A). Similarly, treatment of subcutaneous and omental adipose tissue explants showed with leptin at 100 ng/mL for 20 hours showed no effect on chemerin secretion and protein levels (Fig. 4B–D).

Correlations of Chemerin with Insulin in the Fasting state

Correlations of chemerin with insulin were also evaluated in the fasting state. In the beginning of the fasting period, the standardized regression coefficient between chemerin and insulin was =0.74 (p=0.11) while after 4 days of fasting it was =−0.7 (p=0.86). Regression of the change of insulin levels on chemerin levels also yielded a non-significant association (=0.24, p=0.35)

Discussion

Previous studies in mice have demonstrated that chemerin is a molecule almost exclusively expressed in adipose tissue and liver [8, 10]. Furthermore, studies in humans have evaluated either the expression of chemerin in some tissues/cells [31] or in just adipose tissue [32], but a relative comparison of adipose tissue expression to all other tissue types has not been done. We thus comparatively evaluated for the first time chemerin expression in several human tissue types with that of adipose tissue, by use of human tissue mRNA array. While liver still displays an increased relative expression of chemerin in humans, fat seems to express less chemerin when compared to other tissues such as pancreas and the adrenal gland. Furthermore, upon evaluation of the receptor of chemerin, CMKLR1, we show that it is almost evenly dispersed through multiple human tissues including fat, lung, the adrenal gland, and the duodenum. Furthermore, it appears that the highest expression of CMKLR1 levels are in tissues involved in inflammatory or immune processes such as the lymph nodes, spleen, and the vena cava. This data suggest that chemerin in humans might not be an adipocytokine and may rather play a more prominent role in immune/inflammatory response. The latter is consistent with initial studies demonstrating chemerin as a chemoactractant signal in psoriatic skin lesions [2].

Subsequently, we studied the physiology of chemerin in humans, specifically evaluating for any potential day/night variation of chemerin circulating levels and for chemerin's response to starvation and leptin administration in humans. We demonstrate herein, for the first time

in humans, that, in contrast to leptin, chemerin does not show a significant day/night oscillation. Although no prior human studies have evaluated this, a 20 hour long study of mice with free access to food and water, and blood drawn every 4 hours, has previously demonstrated that chemerin exhibits a day/night pattern with a trough exhibited during the night and a peak during the day [4]. This is another property of chemerin's physiology that we fail to replicate in humans. The human transcriptome displays a day/night variation [33] and many adipokines, such as leptin and high molecular weight adiponectin, display a diurnal variation in humans [34, 35]. Interestingly, both the pancreas and liver, which we demonstrate to be one of the prominent sites of chemerin expression, have a vast number of genes, such the ones encoding proteins essential for glucose metabolism, that express a circadian pattern [36]. In our study we demonstrated absence of any significant day/night oscillation in chemerin circulating levels. We could thus speculate that either chemerin gene expression is not regulated in a circadian fashion in the pancreas and the liver, or that constant, non-circardian secretion from other tissues masks such a pattern. Further studies are encouraged to elucidate this aspect of chemerin's phsysiology.

We also demonstrate, for the first time in humans, that fasting dramatically affects the levels of circulating chemerin. Fasting for 72-hour reduces chemerin levels to ~42% of that seen during the isocaloric fed state. This suggests that chemerin demonstrates an acute response to changes in energy homeostasis such as acute caloric restriction. Interestingly chemerin is highly expressed in tissues that are either direct nutrient sensors, such as the pancreas, or in tissues important for relaying nutrient availability such as the adrenal gland, which produces catecholamines during energy deprivation, and the liver. Therefore, chemerin might be another signal of nutrient status, directed towards the parts of the immune tissues that are expressing the highest CMKLR1, indicating prolonged energy deprivation is occurring, and therefore decreased activity or energy expenditure from those tissues is required. The latter however is only a reasonable speculation derived from chemerin's expression patters as well as its response to acute energy deprivation; further translational studies are needed to confirm this. Although sTNFRII levels did not change with fasting, this does not exclude chemerin-induced immune modification in a TNF-a independent fashion.

Chemerin's response to caloric restriction is similar to that seen by other hormones important in energy homeostasis, such as leptin [35]. Leptin is a key molecule signaling information on the amount of energy stored in the adipose tissue and has been shown to be a regulator of metabolic and neuroendocrine adjustment to starvation [20, 35]. Previous studies from our group have demonstrated that the decrease of leptin levels observed during acute energy deprivation is causally linked to the decrease of other hormones such as luteinizing hormone (LH) [18, 35], while replacement of leptin to physiologic levels corrects starvation-induced changes of neuroendocrine systems [35, 37]. Cross sectional studies have also demonstrated a statistically significant correlation between circulating chemerin and leptin levels in lean, overweight and type-2 diabetic humans [12]. No study to date has evaluated for any causal relationship between leptin and chemerin. Given the fact that in our study fasting had the same effect on the direction of change in both chemerin and leptin circulating levels we wanted to further evaluate for potential regulation of chemerin circulating levels by leptin. We thus performed an interventional cross-over study of metreleptin and placebo administration in the fasting state on the same subjects that we utilized to study for potential day/night variability of chemerin's secretion. We demonstrated that metreleptin replacement has no effect on chemerin levels, implying the lack of any causal relationship between leptin and chemerin. This was also confirmed by our escalating metreleptin dose experiment where administration of low, physiological, and supraphysiological doses of metreleptin during the fed state failed to elicit any dose-response relationship between administered metreleptin dose and measured circulating chemerin levels. Furthermore, we analyzed the effect of 100 ng/mL leptin treatment on subcutaneous and omental adipose tissue explants. Chemerin

levels in the supernatant of both subcutaneous and omental fat tissue as well as its protein expression levels in the subcutaneous adipose tissue were unaltered after overnight treatment with leptin. We thus can report with confidence that leptin does not affect directly or indirectly circulating chemerin levels or expression, suggesting that a mechanism other than leptin controls the acute response of chemerin to decreased energy intake.

Insulin is another hormone that has been suggested to affect chemerin levels in ex vivo human studies. Specifically prolonged insulin infusions increase chemerin secretion in human adipose tissue explants [32]. Although the drop in chemerin levels observed during the fasting state could theoretically be explained by the expected drop in insulin levels, failure of chemerin levels to increase with meals, when insulin levels are expected to increase, surrogates against insulin secretion regulating chemerin levels in vivo. In addition, in our study, chemerin and insulin levels were not correlated in the fasting state at baseline or at day 4, and there was no significant association in regression of the change of insulin levels between fed and fasting, on chemerin levels. Future studies utilizing long term insulin administration, possibly through hyperinsulinemic euglycemic clamps, are needed to definitively rule in or out an effect of dropping insulin levels on chemerin during energy deprivation.

Proinflammatory cytokines such as TNF- have been demonstrated to induce chemerin production *in vitro* and *in vivo* [4, 5]. Production of TNF- by peripheral blood mononuclear cells increases during acute energy deprivation, while spontaneous TNF- production has been noted in patients with anorexia nervosa which is a model disease of chronic energy deprivation[38, 39]. We thus wanted to evaluate whether the decrease of circulating chemerin observed in our study during acute energy deprivation could be attributed to a starvation-induced elevation of circulating TNF- levels. We thus measured sTNFRII, a reliable marker of TNF- system activity [40, 41], in the subjects undergoing acute energy deprivation. Although we did not measure TNF- directly, it has been demonstrated that sTNFRII, is a more easily and readily detected marker of activation of the TNF- system that is highly correlated with TNF- levels [42]. Levels of sTNFRII were unaltered by a 72-hour fast, indicating that the TNF system is not likely responsible for chemerin's changes during short term energy deprivation.

Our study has several strengths: this is the first study in humans examining tissue expression of chemerin and its receptor. It is also the first study in humans to evaluate for day night variability patterns of chemerin secretion and its association with short term changes in energy homeostasis. The temporal resolution of our sampling intervals is high, enabling us to infer the absence of any potential day/night variation patterns with great confidence. In addition, methods were fully standardized and the subjects were seen at the same period of their menstrual cycle, received the same isocaloric diet, were instructed to keep their exercise levels stable and were exposed to the same light/dark intervals throughout the study. Furthermore the randomized cross-over design of this study enables us to control for potential confounding. The 8 week interval between study visits enabled adequate wash out of the medication as well as restoration of the subject's hematocrit. The four day stay in the GCRC enabled proper standardization of the study conditions. And finally the use of multiple study groups as well as the data from ex vivo experiments using tissue explants strongly confirms the findings.

One limitation of this study is the small sample size (n=11) of the physiology studies. However, we overcame this limitation by the frequent sampling approach and the cross-over design of the study, which increases study power. This or similar study samples have been proven to be adequate to detect leptin day/night variation patterns and leptin replacement was able to restore hormonal levels that the 4 day fasting had induced as previously

described [25]. Also, leptin served as a positive control in this study by demonstrating significant day/night rhythm as well as significant effects due to fasting. Thus, the lack of statistically significant changes in the chemerin day night variation could not be attributed to lack of power. Similarly, we confirmed some important findings with other experiments including *ex vivo* adipose tissue leptin treatment. Furthermore, this study is limited by the use of only female subjects for the analysis of Day/Night variation and effect of acute energy deprivation, as well as a dosage response to leptin. Future studies must be carried out in a male population to confirm our results are not limited by gender.

In conclusion, our findings show, for the first time, that chemerin has a different tissue-specific expression pattern compared to rodents, that it is distributed throughout several human tissues with higher expression in pancreas and liver suggesting that it may not be functioning as an adipocytokine in humans. Its receptor, CMKLR1, displays greatest expression in immune tissues such as the spleen and lymph nodes. Furthermore, we demonstrated that, contrary to rodents, chemerin does not exhibit a day/night variation in humans, but is responsive to acute changes in energy homeostasis. We thus recommend clinical studies investigating human circulating chemerin should consider the fasting state of subjects but not necessarily the time of day during blood draws. We also demonstrated that the decreasing levels of chemerin due to starvation are mediated through leptin or the TNF-system. These data contribute towards elucidation of chemerin biology in humans and suggest that chemerin physiology in humans is different from rodents and extreme caution should be applied to any attempt to translate findings regarding chemerin from rodent models to human physiology and pathophysiology. Further studies are needed to elucidate the role of chemerin and its regulation in humans.

Acknowledgments

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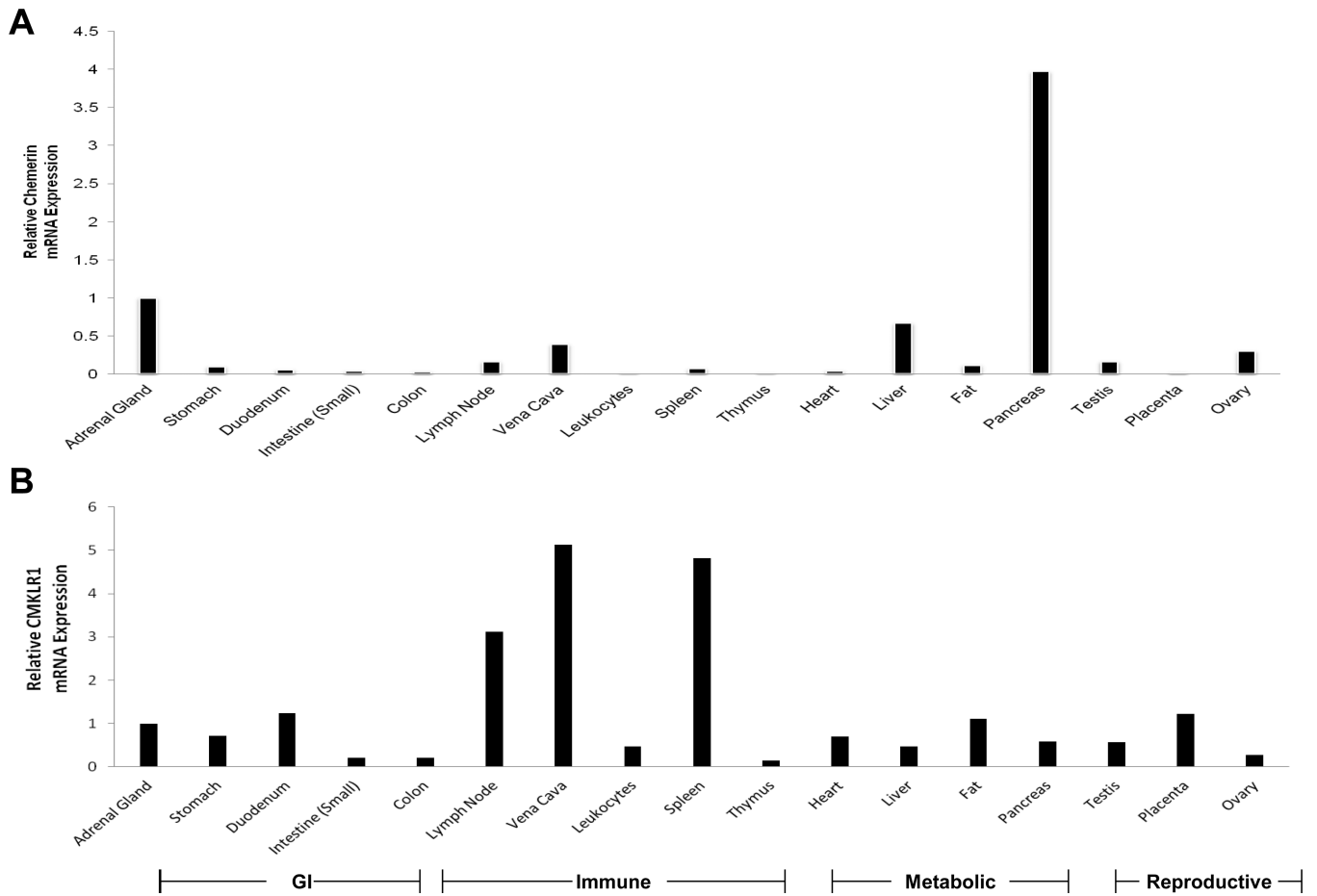
A

B

Figure 1. Tissue mRNA Expression of Chemerin and CMKLR1
Relative mRNA expression of Chemerin (A) and CMKLR1 (B) in human tissues utilizing
Human mRNA Tissue Array. Specific tissue type for each corresponding bar is labeled in
the x-axis of each graph and a legend to group tissues by function in relation to both graphs
A and B is located at the bottom of the figure. Methods of experiment are described in detail
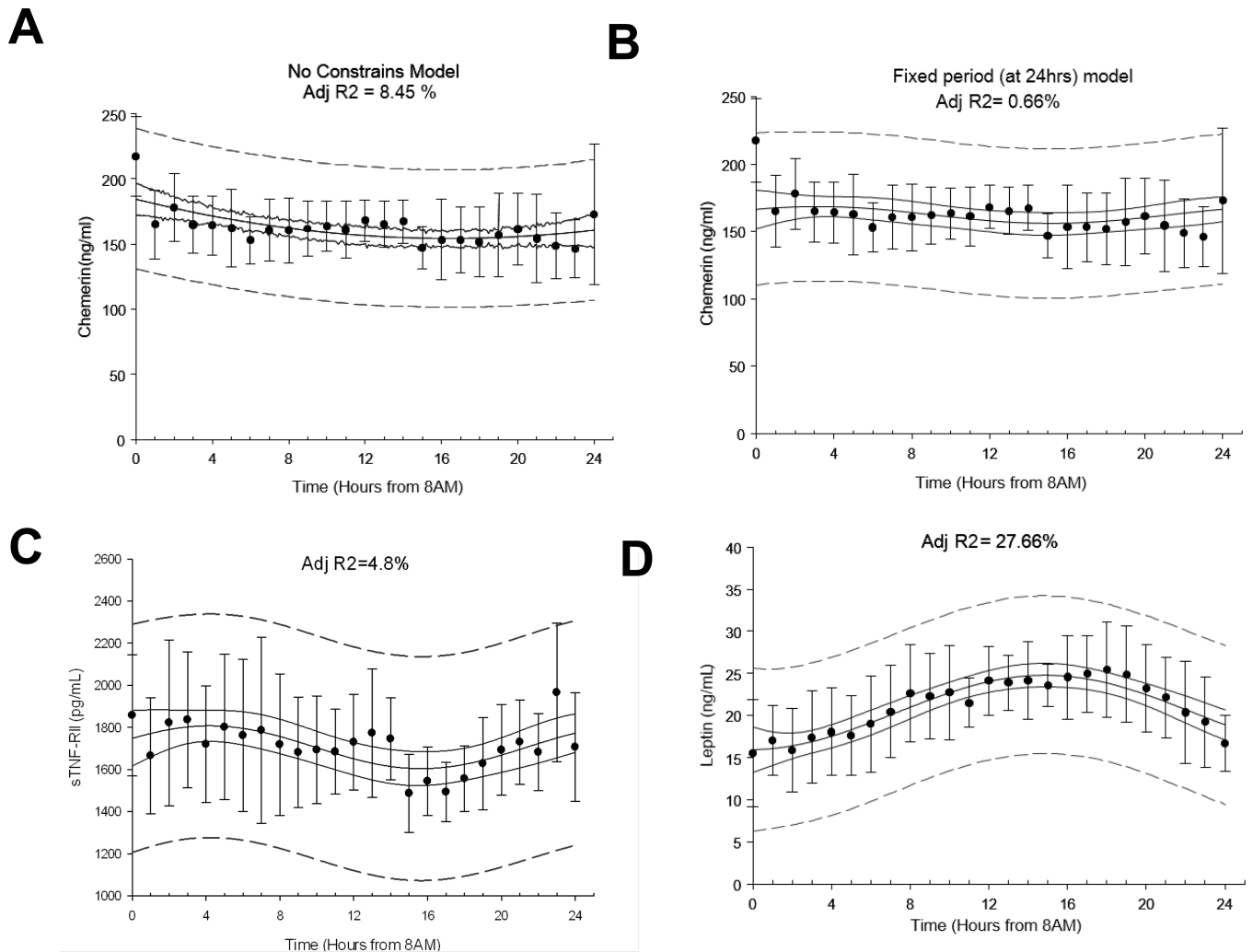in Experimental Procedures section.

A

No Constrains Model
Adj R2 = 8.45 %

B

Fixed period (at 24hrs) model
Adj R2= 0.66%

C

Adj R2=4.8%

D

Adj R2= 27.66%

Figure 2. Day/Night Variation of Chemerin
Average 24-hour (A) chemerin (ng/ml) with no constrains, (B) chemerin (ng/ml) with 24-hour fixed period, (C) sTNFRII (pg/ml) and (D) leptin (ng/ml) levels (non-linear adjusted R^2 is displayed on top center of each panel). Error bars represent SD, continuous line represents 95% confidence band, and dashed line represents 95% prediction bands. Adj-R^2, Adjusted coefficient of determination.
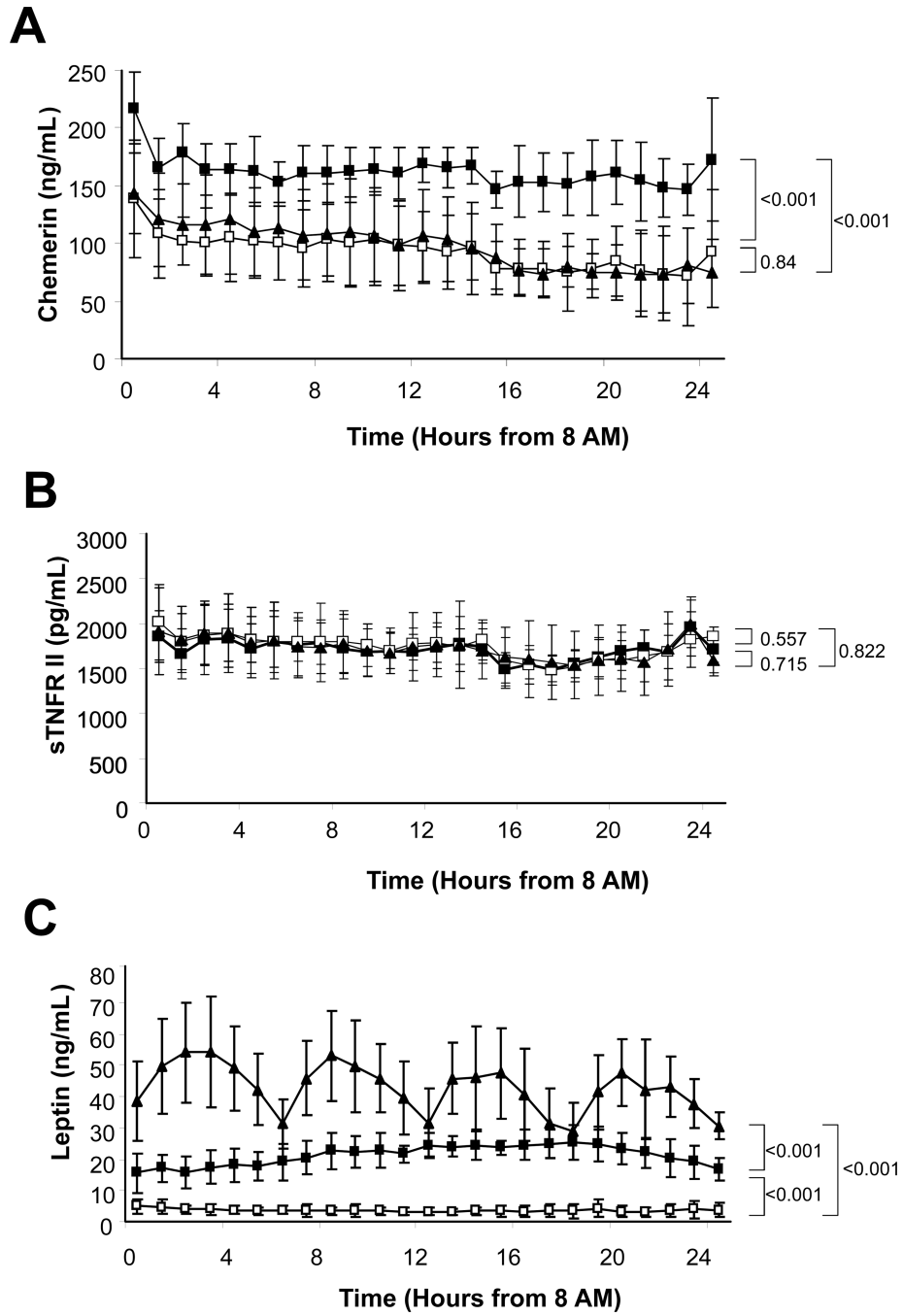
A

B

C

Figure 3. Effects of Acute Energy Deprivation
Average 24-hour (A) chemerin (ng/ml), (B) sTNFRII (pg/ml), and (C) Leptin (ng/ml) levels of fed state ( ), fasting with placebo administration ( ), and fasting with metreleptin administration ( ). P-values are shown next to brackets.
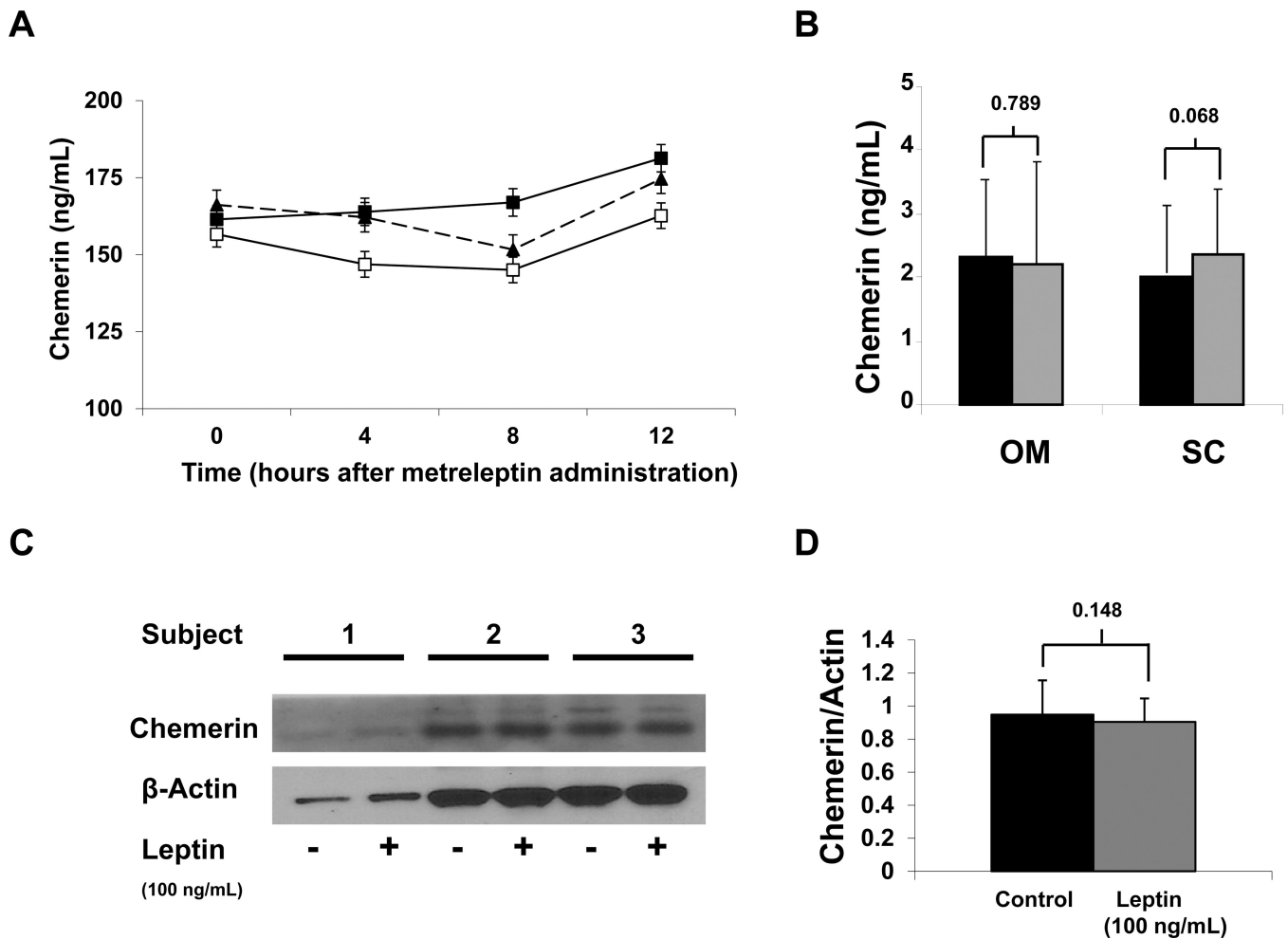
A

B

C

D

Figure 4. Leptin Effects on Chemerin
Average 16-hour (A) chemerin (ng/mL) levels after treatment with three doses of leptin (0.01, 0.1, and 0.3 mg/Kg). Chemerin levels in supernatant (B) with (■) or without (□)100 ng/mL leptin treatment for 20 hours in subcutaneous and omental adipose tissue. Chemerin protein expression (C – D) in subcutaneous adipose tissue after leptin treatment. (B–D) Results are the mean of 3 experiments. Data were analyzed by paired T-Test. Values are means +/− SD.

Table I

Baseline Characteristics vs. Day 4 of Energy Deprivation

Characteristics and chemerin levels at 8 AM on Day 1 and Day 4 of fed state, 72-hour fasting with placebo administration, and 72-hour fasting with metreleptin administration. Data were analyzed by ANOVA for repeated variables.

	Fed State		Fasting + Placebo		Fasting + Leptin		p trt	p time	p trt*time
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4			
Body weight (kg)	57.1 ± 6.5	57.9 ± 6.4	56.9 ± 7.5	55.4 ± 6.9	56.5 ± 6.4	$54.4 \pm 5.8^*$	0.002 (0.001)	0.033 (0.033)	0.033 (0.048)
BMI (kg/m^2)	21.8 ± 2.1	22.2 ± 2.1	21.9 ± 2.7	21.4 ± 2.6	21.7 ± 2.7	$20.9 \pm 2.6^*$	0.001 (0.013)	0.021 (0.021)	0.019 (0.031)
Insulin (μIU/ml)	5.7 ± 1.8	8.1 ± 2.8	6.1 ± 2.0	$1.2 \pm 0.6^*$	6.9 ± 3.3	$1.2 \pm 0.9^*$	<0.001 (<0.001)	<0.001 (<0.001)	<0.001 (<0.001)
Leptin (ng/ml)	11.2 ± 4.5	16.7 ± 3.3	14.7 ± 6.3	$2.8 \pm 0.8^*$	12.5 ± 4.6	$29.6 \pm 5.4^*$	<0.001 (<0.001)	0.282 (0.282)	<0.001 (<0.001)
sTNFRII (pg/ml)	1753.75 ± 170	1706.5 ± 257	1775.9 ± 339	1810.8 ± 230	1596.6 ± 178	1865.9 ± 158	0.726 (0.726)	0.644 (0.644)	0.651 (0.651)
Chemerin (ng/ml)	164.6 ± 37.2	155.0 ± 42.4	161.1 ± 34.0	$56.9 \pm 26.6^*$	151.0 ± 34.5	$50.9 \pm 8.6^*$	<0.001 (0.003)	<0.001 (<0.001)	0.006 (0.027)

Values are means +/− SD. Means with * indicate significant difference between day 1 and day 4 within a state. P trt demonstrates significance of intervention (fed, fasting, leptin); p time demonstrates significance of time between days; p trt*time demonstrates significance of intervention and time interaction.