Circadian Rhythm of Serum Vaspin in Healthy Male Volunteers: Relation to Meals

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Context: Visceral adipose tissue-derived serine protease inhibitor (vaspin) is a novel adipokine with insulin-sensitizing effects. However, the physiological role for vaspin in human metabolic regulation remains to be established.

Objective: We studied the 24-h profiles of circulating vaspin concentrations in relation to meal ingestion in normal adults.

Design: Blood samples were drawn 39 times throughout a 24-h period from 10 healthy male subjects provided with meals on a fixed schedule. On a separate day, four subjects were fasted and then provided with an unexpected meal to clarify the effect of meal consumption on serum vaspin levels. Serum vaspin concentrations were determined by ELISA.

Results: Serum vaspin levels were highest in the early morning before breakfast and fell to trough levels within 2 h after breakfast. Serum vaspin levels also showed a preprandial rise and postprandial fall at lunch and dinner, although at lesser degrees than at breakfast. Intermeal vaspin concentrations reached a nadir in the mid-afternoon and showed a nocturnal rise, with peak nighttime vaspin levels being approximately 250% of nadir levels. Unscheduled food ingestion after a prolonged fast significantly reduced serum vaspin levels, suggesting that energy intake itself has a suppressive effect on serum vaspin levels. The diurnal pattern of serum vaspin concentrations was exactly reciprocal to that of insulin and of glucose.

Conclusion: Serum vaspin levels have a meal-related diurnal variation, suggesting a role for vaspin in metabolic regulation. However, the reciprocal relationship between serum vaspin and insulin may negate the importance of vaspin as an physiological insulin sensitizer. (***J Clin Endocrinol Metab* 95: 1869–1875, 2010**)

Cumulating evidence suggests that adipose tissues secrete various biological proteins, so-called adipokines, thereby actively regulating body weight homeostasis, glucose and fatty acid metabolism, and vascular functions (1). Leptin, mostly derived from white adipose tissue, acts in the hypothalamus to regulate food intake and energy expenditure (2). Deficiencies of leptin cause severe obesity and hyperphagia in humans and rodents (3, 4), suggesting that leptin is a pivotal regulator of appetite and body weight. Adiponectin is another important adipokine that increases insulin sensitivity and stimulates fatty acid oxidation in skeletal muscle and has antiinflammatory effects in blood vessels (5–7). Decreased adiponectin levels account for obesity-related insulin resistance and vascular dysfunction, because these abnormalities are reversed by adiponectin treatment (8, 9). In contrast, adi-

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Abbreviations: AUC, Area under the curve; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance.

pocytes also secrete proinflammatory adipokines such as resistin (10), tumor necrosis factor-α (11), and IL-6 (12), which contribute to the development of insulin resistance in the obese state.

Vaspin was first isolated from the visceral white adipose tissue of the Otsuka Long-Evans Tokushima fatty (OLETF) rat, an animal model of visceral obesity and type 2 diabetes mellitus (13). Although vaspin has structural homology to the serine protease inhibitor (serpin) family (13), serpin activity by vaspin has not yet been demonstrated. Vaspin expression in the visceral adipose tissue of prediabetic OLETF rats increases with age from 6 to 30 wk old and thereafter decreases with progression of diabetes mellitus. Interestingly, the decreased vaspin levels in the diabetic state are reversed by exercise or treatment with insulin and the insulin sensitizer thiazolidinedione, each of which improves hyperglycemia. Furthermore, administration of vaspin significantly improves glucose intolerance and insulin resistance in diet-induced obese mice (13). Therefore, vaspin has been suggested to be a visceral adipose-derived adipokine with insulin-sensitizing effects.

In humans, serum vaspin concentrations positively correlate with age, body mass index (BMI), and insulin sensitivity impairment, although these relationships are abrogated in type 2 diabetic patients (14, 15). On the other hand, serum vaspin concentrations are decreased by metformin treatment in subjects with polycystic ovary syndrome but increased by exercise training (15, 16). Therefore, a role for vaspin in human metabolic regulation is unclear at present.

Several hormones and adipokines have specific diurnal variations, from which one can assume their physiological roles in metabolic regulation. For example, circulating insulin levels increase in the postprandial state and decrease in the fasted state (17). The diurnal variation of insulin is compatible with the glucose-lowering effects of insulin. A postprandial rise in circulating insulin levels may stimulate glucose disposal after food ingestion, whereas decreased insulin levels during a fast prevent hypoglycemia. The appetite-stimulating hormone ghrelin shows a dramatic premeal surge, suggesting its role as a meal initiator (18). Conversely, the diurnal rhythm of leptin shows a nocturnal rise that may have an appetite-suppressing effect during the night (19).

In the present study, we investigated the diurnal variation of serum vaspin levels in healthy volunteers. Serum vaspin concentrations displayed a circadian rhythm, along with a preprandial rise and postprandial fall. Furthermore, unscheduled meal intake decreased serum vaspin concentrations, indicating that circulating vaspin concentrations were negatively regulated by energy intake.

Subjects and Methods

Subjects

A total of 10 healthy male subjects, aged 18–30 yr, were recruited through local website advertising. Five lean (BMI 17–23 kg/m^2) and five obese (BMI 27–40 kg/m^2) subjects were recruited. We defined obesity as BMI of 25 kg/m^2 or higher according to Asia-Pacific obesity criteria proposed by the Western Pacific Regional Office of the World Health Organization (20). All subjects had a stable body weight for at least 3 months before the study. Subjects had no history of diabetes, hypertension, other chronic medical or psychiatric illnesses, gastrointestinal surgery, regular medication, smoking, or heavy alcohol consumption (more than two drinks a day) and did not regularly and intensely exercise (defined as >30 min aerobic exercise three times per week).

Subjects signed a written informed consent and completed a self-administered questionnaire that included demographic characteristics, general health status, alcohol and smoking history, and current medications. Blood pressure, height, weight, and waist circumference were measured by standard protocols. To screen eligibility, subjects were subjected to a routine physical examination, measurement of vital signs, electrocardiography, and routine laboratory tests including a complete blood count, electrolytes, fasting glucose, lipid profiles, and a liver function test. None of the subjects displayed any abnormality in the screening tests (Table 1).

Study design

Before admission, eating behaviors of the subjects were assessed by a dietitian using a 3-d diet diary. Subjects were educated to ingest calories to maintain weight stability for 1 wk before the study. Their diets consisted of 60% carbohydrates, 25% fats, 15% proteins, and macronutrient contents that approximate the average Korean diet. At the end of the 1-wk diet period, subjects were admitted to the Asan Clinical Research Center at 1700 h 1 d before the study day and were provided with dinner. An indwelling iv catheter was placed. After an overnight fast, blood was drawn into EDTA-coated tubes and serum-separating tubes containing aprotinin (250 kallikrein inhibitor units; Sigma-Aldrich, Seoul, Korea) at 30-min intervals from 0700–2100 h and then hourly until 0700 h in the next morning (24 h total). During the study day, the subjects had breakfast at 0800 g, lunch at 1230 h, and dinner at 1800 h, which consisted of 60% carbohydrates, 25% fats, and 15% proteins. Subjects spent their time in bed, except for during waste elimination. Lights were off from 2200–0600 h.

On a separate day at least 2 wk after the first admission, four (two lean and two obese) subjects were readmitted. Instead of a three-meal schedule as in the first study, the four subjects were fasted and allowed to have only water until they were given a meal at 1500 h without any notice.

Plasma was separated immediately by centrifugation at 4 C. Serum was separated by centrifugation at 4 C after clotting for 20 min at room temperature. Blood samples were separated into aliquots and stored at −70 C until they were assayed. The study was approved by the Institutional Review Boards at the Asan Medical Center (Seoul, Korea) and carried out in accordance with the principles of the Helsinki Declaration.

TABLE 1. Demographic and biochemical characteristics of subjects

	Total (n = 10)	Lean (n = 5)	Obese (n = 5)
Age (yr)	23 ± 1	23 ± 1	23 ± 2
BMI (kg/m^2)	25.8 ± 1.5	21.9 ± 0.6	29.8 ± 1.4
Waist (cm)	89.4 ± 3.4	80.4 ± 2.1	98.4 ± 2.8
Systolic BP (mm Hg)	122 ± 9	119 ± 8	124 ± 10
Diastolic BP (mm Hg)	74 ± 5	74 ± 4	75 ± 6
Fasting glucose (mmol/liter)	5.1 ± 0.1	5.0 ± 0.2	5.2 ± 0.2
Total cholesterol (mmol/liter)	4.5 ± 0.2	4.2 ± 0.2	4.8 ± 0.1
Triglyceride (mmol/liter)	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
LDL-cholesterol (mmol/liter)	2.9 ± 0.2	2.6 ± 0.2	3.3 ± 0.2
HDL-cholesterol (mmol/liter)	1.2 ± 0.04	1.2 ± 0.06	1.2 ± 0.04
Insulin (pmol/liter)			
Fasting	99.0 ± 22.6	72.5 ± 8.9	126.3 ± 43.0
24-h AUC	7159.9 ± 1427.8	6104.5 ± 385.6	8215.4 ± 2910.2
HOMA-IR	3.08 ± 0.64	2.22 ± 0.28	3.95 ± 1.17
Leptin (ng/ml)			
Fasting	3.50 ± 0.87	1.97 ± 0.63	5.03 ± 1.35[a]
24-h AUC	119.9 ± 21.0	90.6 ± 19.9	149.2 ± 34.0[a]
Vaspin (ng/ml)			
Fasting	0.56 ± 0.12	0.43 ± 0.08	0.69 ± 0.22
24-h AUC	9.1 ± 1.6	7.1 ± 0.9	11.2 ± 2.9
Cortisol (μg/dl)	11.50 ± 0.53	12.00 ± 0.66	11.00 ± 0.51

Data are average ± SEM. BP, Blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

[a] $P < 0.05$ *vs.* lean group.

Assays

Fasting plasma glucose, total cholesterol, triglyceride, and high-density lipoprotein cholesterol levels were measured by enzymatic methods using an autoanalyzer (Hitachi E170; Hitachi, Ltd., Tokyo, Japan). Serum vaspin levels were assayed using a sandwich ELISA kit (AdipoGen, Seoul, Korea) according to the manufacturer's instructions (15). The lower and upper limits of detection were 0.016 and 1 ng/ml respectively. The intra- and interassay coefficients of variation were 1.3–3.8 and 3.3–9.1%, respectively. All samples from a single individual were run in duplicate.

Commercial RIA kits were used for the measurement of plasma insulin and leptin levels (Linco Research, St. Charles, MO). Plasma cortisol levels were determined using RIA (Siemens Healthcare Diagnostics, Los Angeles, CA). The homeostasis model assessment of insulin resistance (HOMA-IR) was used to evaluate insulin resistance (21).

Statistical analysis

Data are presented as mean ± SEM. The 24-h integrated area under the curve (AUC) values were calculated with use of the trapezoid rule. Serum vaspin concentrations at different times over the 24-h period were compared using the repeated-measures ANOVA. The differences of vaspin AUC values between two feeding studies were assessed by the Wilcoxon signed rank test. Fasting and AUC values of various hormones between lean and obese groups were compared using the Mann-Whitney *U* test. Spearman's correlation coefficient analysis or multiple linear regression analysis was performed to evaluate the association of two variables. SPSS 13.0 software (SPSS, Chicago, IL) was used for statistical analysis. A P value < 0.05 was considered statistically significant.

Results

Serum vaspin concentrations rose 1–2 h before the onset of each meal by average values of 0.15 ng/ml for breakfast, 0.09 ng/ml for lunch, and 0.13 ng/ml for dinner. Serum vaspin levels reached trough levels within 2 h after food intake initiation (Fig. 1A). Changes in vaspin levels before and after a meal was greatest for breakfast and gradually declined for lunch and dinner (breakfast 0.34 ± 0.09 ng/ml, lunch 0.16 ± 0.04 ng/ml, and dinner 0.12 ± 0.07 ng/ml). Intermeal vaspin concentrations reached a nadir in mid-afternoon and showed a nocturnal rise, with peak nighttime vaspin levels that were about 250% higher than nadir levels (nadir 0.22 ± 0.06 ng/ml, nocturnal peak 0.53 ± 0.10 ng/ml, $P < 0.005$).

With regard to its temporal relationship to meals, the 24-h time course of serum vaspin concentrations was reciprocal to that of insulin ($r = -0.578$; $P < 0.01$; Fig. 1, A and B). Whereas serum vaspin rose before each meal time and declined after meal ingestion, insulin levels increased within 30 min after initiation of meal consumption by 4.7- to 8.3-fold from premeal trough levels and slowly returned to trough levels before the next meal. In addition, serum vaspin levels showed a nocturnal rise, whereas serum insulin levels remained low during sleeping time. Similarly, there was a negative relationship between serum vaspin and glucose concentrations (Fig. 1, A and C; $r = -0.675$; $P < 0.01$). However, no significant correlation was found between circulating vaspin and cortisol

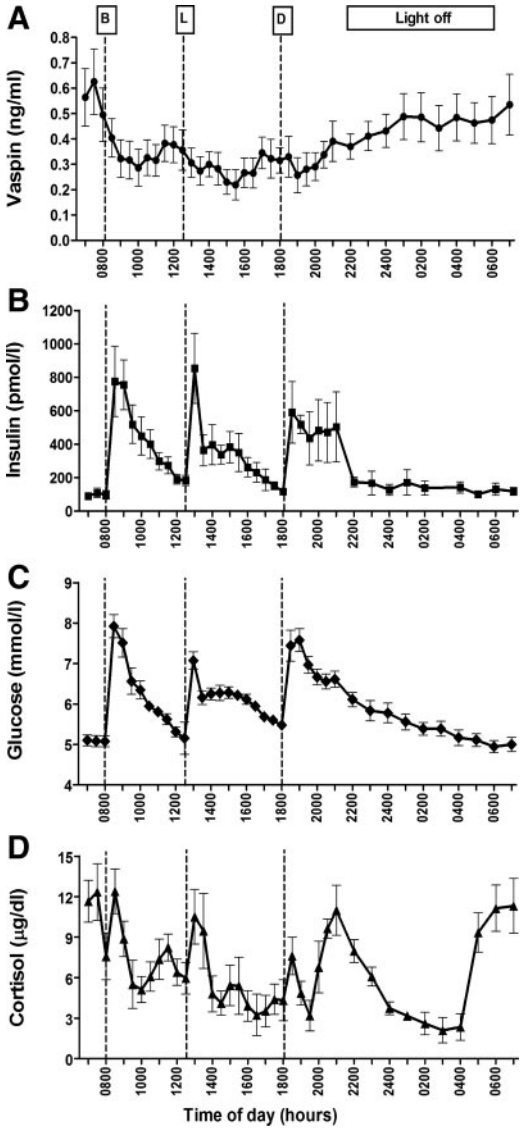

FIG. 1. Average serum vaspin (A), insulin (B), glucose (C), and cortisol (D) concentrations during a 24-h period in 10 human subjects consuming breakfast (B), lunch (L), and dinner (D) at the times indicated (0800, 1230, and 1800 h, respectively).

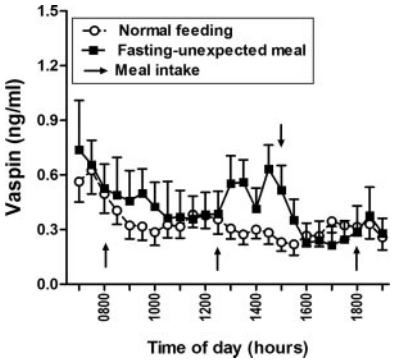

FIG. 2. Comparison of the average serum vaspin concentrations between the normal feeding study and the fasting-unexpected meal study. In the normal feeding study, subjects were provided breakfast, lunch, and dinner at 0800, 1230, and 1800 h. In the fasting-unexpected meal study, subjects were fasted until a meal was provided without notice at 1500 h.

levels (r = 0.273; $P = 0.093$; Fig. 1, A and D). Multiple linear regression analysis also revealed that serum vaspin levels were negatively affected by serum insulin levels ($\beta = -0.158$; $P < 0.001$) but not by cortisol levels ($\beta = 0.004$; $P = 0.107$).

To further clarify the relationship between serum vaspin levels and meal ingestion, serum vaspin profiles were investigated in a different feeding paradigm, in which four subjects were fasted during the usual breakfast and lunch times. They were then given an unexpected meal in the mid-afternoon (at 15:00). Fasting increased circulating vaspin concentrations (Vaspin AUC from 0700 to 1500: normal feeding 2.23 ± 0.47 ng/ml *vs.* fasting 3.86 ± 0.54 ng/ml, $P < 0.05$). Furthermore, unexpected meal ingestion significantly decreased serum vaspin levels (before meal 0.64 ± 0.18 ng/ml, 2 h post meal 0.21 ± 0.10 ng/ml, $P < 0.05$, Fig. 2), suggesting that serum vaspin levels were suppressed by unexpected meal ingestion.

Obese subjects had higher fasting and 24-h AUC leptin levels compared with lean subjects ($P < 0.05$, Table 1). Both the fasting and 24-h AUC vaspin values tended to be higher in obese subjects than in lean subjects, as did the insulin levels. However, these differences did not reach a statistical significance due to the small number of subjects and individual variability (Table 1). On the other hand, there was no significant association between fasting serum vaspin concentrations and insulin resistance index HOMA-IR (r = 0.164; $P = 0.65$). Diurnal variations of vaspin, insulin, and leptin were compared between lean and obese subjects (Fig. 3, A–C). Obese subjects had a tendency of increased vaspin, insulin, and leptin levels over the 24-h period. Diurnal rhythms of vaspin, insulin, and leptin were not altered in obese subjects, although meal-related changes in serum vaspin levels were augmented at lunch but blunted at dinner (Fig. 3A).

The average morning fasting vaspin values (sampled at 0700 and 0730 h) were well correlated with the 24-h AUC values (r = 0.818; $P < 0.005$; Fig. 4), suggesting that morning fasting vaspin values could be a good surrogate for the vaspin 24-h integrated AUC values.

Discussion

Hormones are characterized by biological rhythms, which may reflect their physiological roles. In this study, we first showed that human serum vaspin concentrations increase before each meal and decrease after eating. The preprandial rise and postprandial fall in 24-h serum vaspin profiles were similar to the diurnal rhythm in plasma ghrelin levels (18), although meal-related changes in serum vaspin levels

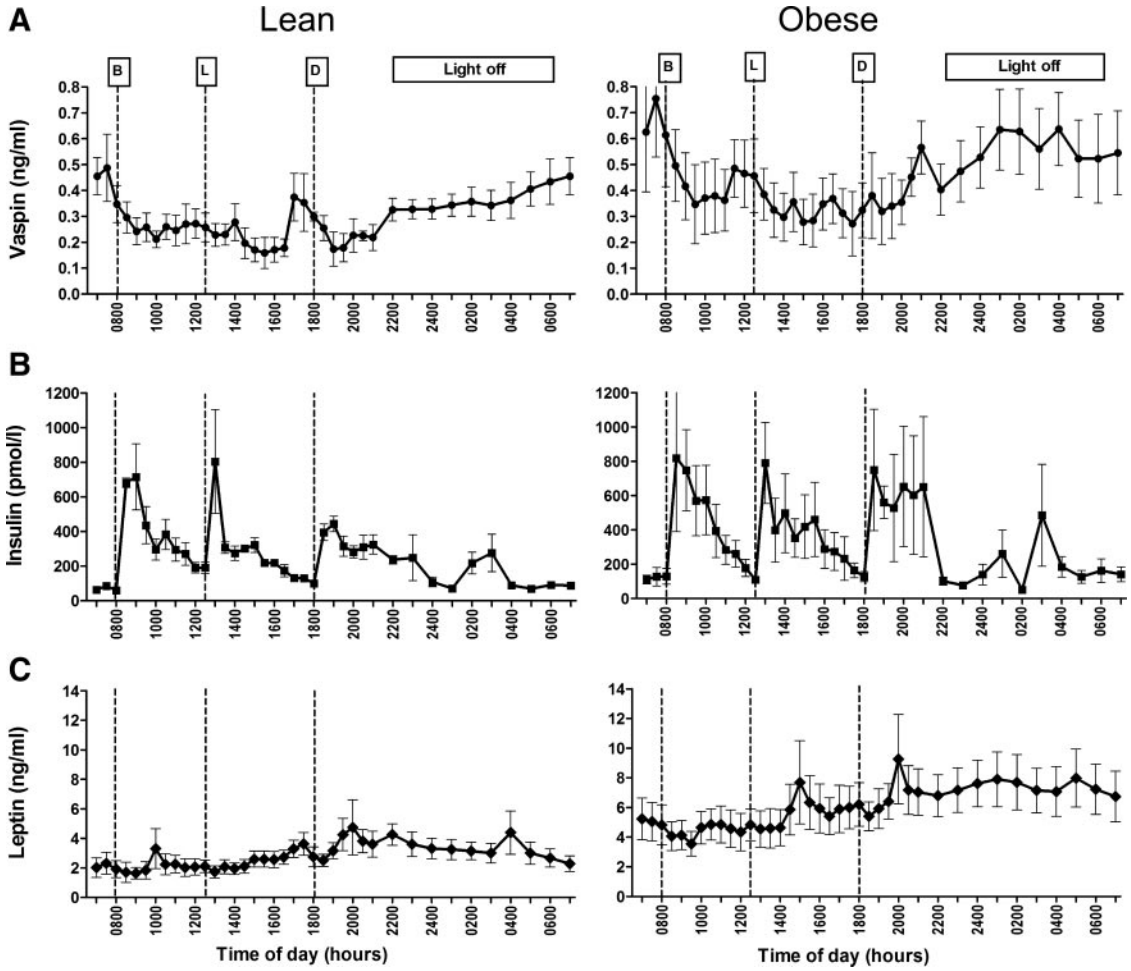

FIG. 3. Diurnal variations of serum vaspin (A), insulin (B), and leptin (C) concentrations in lean and obese subjects consuming breakfast (B), lunch (L), and dinner (D) at the times indicated.

are less dramatic than those of ghrelin. Because the subjects knew the mealtimes in our normal feeding study, a premeal rise in serum vaspin levels might have been an anticipatory response to meals. Therefore, we conducted a fasting-unexpected meal study, in which the subject did not know the meal schedule. During a prolonged fast, the usual morning decrease (from 0800–1000 h) in serum vaspin concentrations tended to be blunted and delayed.

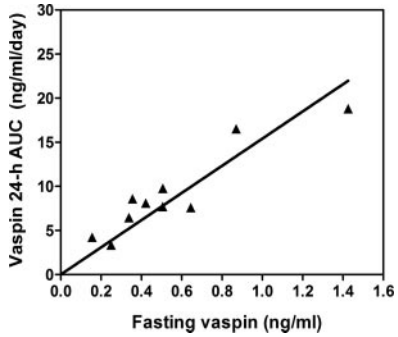

FIG. 4. Correlations between integrated 24-h AUC vaspin values and average fasting serum vaspin values measured at 0700 and 0730 h ($r = 0.818$; $P < 0.005$).

Moreover, vaspin concentrations rose between 1300 and 1500 h, when serum vaspin levels declined after lunch in the normal feeding study. These findings suggest that fasting increases serum vaspin concentrations, although a morning fall in circulating vaspin levels may be controlled by the biological clock to some degree. Consistent with this, meal consumption, whether provided with or without previous notice, significantly reduced serum vaspin levels, confirming that the postprandial decrease in serum vaspin levels may be caused by energy intake itself.

Nutrients or hormones such as insulin and ghrelin may mediate the effects of meal ingestion on serum vaspin levels. Insulin may have a negative effect on serum vaspin levels, because patients on insulin treatment display lower serum vaspin levels (14). Consistently, there was a significant negative correlation between serum vaspin and insulin concentrations in this study. However, insulin treatment increases serum vaspin levels in diabetic OLEFT rats (13) and has no effect on vaspin expression or secretion in *ex vivo* omental fat cultures (16). In our hands, insulin did not change vaspin protein expression levels in 3T3-L1 adi-

pocytes (data not shown). Therefore, it is unlikely that insulin directly acts on the adipocytes to regulate vaspin expression or secretion. Glucose also may not mediate meal-induced suppression in serum vaspin concentrations. A previous study showed that glucose increases vaspin expression and secretion from omental fat pads (16). Moreover, serum vaspin levels are positively associated with hemoglobin A1c in diabetic patients (22). In contrast to these previous findings, we observed a reciprocal relationship between serum vaspin and glucose concentrations in the 24-h profiles of healthy volunteers.

From data in obese diabetic rodent models, vaspin was originally suggested to be an insulin-sensitizing and glucose-lowering adipokine (13), although the molecular mechanisms and action sites have remained unknown. However, we demonstrated here that the 24-h profiles of serum vaspin levels are reciprocal to those of circulating insulin and glucose in humans. These data may raise questions on the role of vaspin as an insulin-sensitizing hormone in humans.

The observed nocturnal increase in serum vaspin levels resembles those of ghrelin and leptin (18, 19). The nocturnal rise of vaspin also resembles the change in nonesterified free fatty acid levels during the night (23). There is a lack of *in vitro* and *in vivo* data on the hormonal regulation of vaspin production and secretion by adipose tissues, making it difficult to ascertain which of these factors, if any, directly regulate the nocturnal rise in vaspin levels in humans. Leptin and vaspin are secreted from white adipose tissue (4, 13). Circulating levels of leptin and vaspin increase with adiposity, whereas weight loss reduces the levels of both (14, 15, 24), suggesting some relationship between the two hormones. Interestingly, we have also found high levels of vaspin mRNA and protein expression in the mouse stomach (Jang, P. G., C. Namkoong, G. M. Kang, and M.S. Kim, unpublished data). Given the similarity in the diurnal rhythms of vaspin and ghrelin, it will be interesting to further investigate the relationships between vaspin and ghrelin. Meanwhile, serum vaspin levels were highest at morning fasting state. A similar early morning rise was seen in plasma cortisol levels. However, there was no significant correlation between vaspin and cortisol levels in the present study.

Consistent with previous reports (14, 15), serum vaspin levels tended to be higher in subjects with a higher BMI, as was also the case for insulin and leptin levels. Obesity itself or obesity-related insulin resistance may increase serum vaspin concentrations, because serum vaspin levels are decreased by metformin treatment and in subjects with high fitness levels (15, 16). In our study, serum vaspin levels were not significantly correlated with HOMA-IR, a surrogate marker of insulin resistance. Thus, insulin resistance may not account for obesity-related increase in serum vaspin levels. Finally, we showed that morning fasting vaspin levels were well correlated with vaspin 24-h AUC values. Thus, a casual measurement of fasting morning vaspin levels can be used to predict the 24-h circulating vaspin concentrations.

In summary, we demonstrated premeal and nocturnal rises and postmeal falls in the serum vaspin concentrations of human subjects. Meal-related changes in serum vaspin concentrations may suggest a role for vaspin in the regulation of nutrient and body weight homeostasis. However, our study is limited by a small sample size. Thus, other confirmatory independent studies may be needed in the future.

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