Circadian Rhythm of Circulating Fibroblast Growth Factor 21 Is Related to Diurnal Changes in Fatty Acids in Humans

Haoyong Yu, Fuzhen Xia, Karen S.L. Lam, Yu Wang, Yujian Bao, Jiali Zhang, Yunjuan Gu, Pengcheng Zhou, Junxi Lu, Weiping Jia, and Aimin Xu

BACKGROUND: Fibroblast growth factor (FGF) 21 is an endocrine factor actively involved in glucose and lipid metabolism in rodents. However, little is known about its physiological function and regulation in humans. This study investigated the diurnal changes in circulating FGF21 concentrations and their association with other metabolic markers in both obese and lean individuals.

METHODS: A total of 36 volunteers were assigned to 2 groups. One group received 3 standardized meals and another group was fasted for 24 h. Blood samples were drawn every 30 min throughout a 24-h period. Circulating FGF21 concentrations were measured with an in-house chemiluminescence immunoassay. The effects of fatty acids on hepatic production of FGF21 were determined by using real-time PCR.

RESULTS: In both the fasting and standardized meals groups, circulating FGF21 began to rise at midnight, reaching a peak in the early morning and then declining to basal concentrations early in the afternoon. Baseline concentrations of circulating FGF21 were much higher in obese individuals than in lean individuals (P < 0.05). However, the magnitude of the nocturnal rise in circulating FGF21 was significantly blunted in obese individuals. The 24-h oscillatory pattern of circulating FGF21 resembled that of free fatty acids and cortisol, but was opposite to the patterns of insulin and glucose. Unsaturated fatty acids induced time-dependent expression of FGF21 mRNA in human hepatocytes.

CONCLUSIONS: These findings support the role of FGF21 as an important metabolic regulator that integrates the circadian rhythm with energy homeostasis in humans. Diurnal rhythms of circulating FGF21 could be partly caused by the oscillation of free fatty acids.

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dation, tricarboxylic acid cycle flux, and gluconeogenesis (8). Furthermore, FGF21 is also involved in lipolysis modulation in white adipose tissue (6, 9) and thermogenesis in brown adipose tissue of neonatal mice (10).

Little is known about the physiological roles and regulation of FGF21 in humans. Previous studies in different ethnic groups have demonstrated an increased concentration of circulating FGF21 in obese individuals (11) and patients with type 2 diabetes (12), dyslipidemia (13), insulin resistance (14), and alcoholic fatty liver disease (15, 16). The increased FGF21 concentrations in obesity are attributable to increases in free fatty acids (FFAs), which can directly stimulate FGF21 expression through the activation of PPARα (17). Hyperinsulinemia during glucose clamping studies has been shown to induce FGF21 expression in human skeletal muscle (18).

The response of circulating FGF21 to fasting and nutritional changes in humans appears to be different from that observed in rodents. In mice, a marked induction of FGF21 expression occurs within several hours in response to fasting (5, 6). However, in humans, starvation for up to 72 h does not affect, or may even cause a slight decrease in, circulating FGF21 (15). A modest increase in circulating FGF21 is detected only after prolonged fasting for 7 days (19). Published data related to the effects of ketogenic diet on circulating FGF21 concentrations in humans are inconsistent (15, 19, 20).

To better understand the metabolic roles of FGF21 and its regulation in humans, we developed a highly sensitive and specific chemiluminescence immunoassay (CLIA) to measure temporal changes in circulating FGF21 concentrations under both fasting and fed conditions over 24 h. Our results demonstrate a characteristic diurnal pattern of circulating FGF21 in humans, with a major nocturnal rise occurring after midnight. In obese individuals, the magnitude of this nocturnal rise is significantly blunted.

Materials and Methods

STUDY PARTICIPANTS

Three cohorts of volunteers were recruited for this study. The clinical characteristics of these study groups are described in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue5. A BMI of ≥25 kg/m² was defined as obese on the basis of Asian-Pacific obesity criteria proposed by the WHO Western Pacific Regional Office. Written consent was obtained from all volunteers. The study protocol was approved by our institutional ethics committee.

For the first study cohort (n = 7), the participants were admitted to the metabolic ward at 1700 and were provided with a standardized dinner. These individuals were then fasted and allowed to have water access only for a 24-h period until 1800 the next day. An antecubital vein was cannulated for blood sampling. Blood samples were taken from individuals in the first cohort at 1800 on day 1 and 0900 and 1800 on day 2. For the second study cohort (n = 10), blood samples were taken from participants every 30 min during the period of 24 h of fasting from 1800 on day 1 to 1800 on day 2. For the third study cohort, 19 volunteers were admitted to the metabolic ward at 0700 and were provided with 3 standardized meals (breakfast at 0800, lunch at 1300, and dinner at 1800). Blood samples were drawn from an indwelling venous catheter in the forearm every 30 min over a period of 24 h.

BIOCHEMICAL ANALYSIS AND IMMUNOASSAY FOR BLOOD SAMPLES

The serum concentrations of human FGF21 were measured with an in-house CLIA, as detailed in the online Data Supplement. Glucose, triglyceride, total cholesterol, LDL cholesterol, and HDL cholesterol concentrations were determined enzymatically on a Hitachi 7600 chemical analyzer (Hitachi). Serum insulin was measured with RIA (Linco Research). Serum concentrations of FFAs and cortisol were quantified by enzymatic colorimetric assay and electrochemiluminescence immunoassay (Roche Diagnostics), respectively.

CELL CULTURE AND IN VITRO ASSAYS

Palmitic, oleic, or linoleic acid were dissolved in ethanol and then mixed with an aqueous solution of fatty acid–free BSA (in DMEM) at 45 °C until homogenous at a molar ratio of 2.5:1. Human HepG2 hepatocytes (ATCC) were grown in DMEM containing 1% FBS for 16 h, followed by treatment with different concentrations of fatty acids for different periods. The conditioned medium was harvested, centrifuged at 13 000g for 5 min to remove cell debris, and then analyzed for FGF21 mRNA concentrations. Total RNA was extracted by using Trizol reagent (Invitrogen) and was then subjected to reverse transcription and quantitative real-time PCR to determine the relative mRNA abundance of FGF21 as previously described (11).

DATA ANALYSIS

Data are expressed as mean (SE). All statistical calculations were performed with the SPSS 11.5 statistical software package. The assumption of normality was tested by using a Kolmogorov–Smirnov test. Comparisons between groups over time were per-
formed with repeated-measures ANOVA. Pearson correlation coefficients were used to evaluate the association between the concentrations of FGF21 and FFA. The diurnal rhythm of serum FGF21 was quantitatively described with cosinor analysis (21). The 95% statistical CIs were determined for the 24-h cosine amplitude (half of the peak-nadir difference), MESOR (midline estimating statistic of rhythm), and acrophase (clock time of maximal value). In all statistical tests, P values <0.05 were considered as significant difference.

Results

DEVELOPMENT OF A HIGH-SENSITIVE CLIA FOR HUMAN FGF21
To quantify the circulating concentrations of human FGF21, we generated antihuman FGF21 antibodies in rabbits by using recombinant full-length protein as an immunogen (Fig. 1A). To confirm the specificity of the antibodies, human serum was subjected to chromatographic purification by using antihuman FGF21 antibodies as a ligand. The affinity-purified FGF21 from human serum was digested by trypsin and analyzed by an Orbitrap Velos mass spectrometer (Thermo). The protein identity was confirmed by comparing the sequences of the 3 peptides derived from trypsin digestion with the published sequence of human FGF21 (NCBI accession number: NP 061986) (Fig. 1B), suggesting that the antibodies recognize native FGF21 in the circulation. The antibodies were then used for the development of an in-house CLIA for measuring human FGF21. The assay did not cross-react with other members of the human FGF family and basic FGF.

We next evaluated the assay by measuring serum FGF21 concentrations in 84 healthy individuals (from 30 to 50 years old) after overnight fasting. Serum FGF21 concentrations displayed a normal distribution (Fig. 1C), with the median concentration of approximately 125 ng/L.

**Fig. 1. Development and validation of CLIA specific to human FGF21.** (A), SDS-PAGE analysis of recombinant human FGF21 purified by affinity chromatography. (B), Representative liquid chromatography–tandem mass spectrometry spectra of native FGF21 purified from human serum using antihuman FGF21 antibody as an affinity ligand. The sequences of the 3 peptides determined by tandem mass spectrometry analysis are identical to the published sequence of human FGF21 as denoted. (C), Distribution of human serum concentrations of FGF21 in 84 healthy Chinese individuals.
TEMPORAL CHANGES IN CIRCULATING FGF21 UNDER FASTING CONDITIONS IN HEALTHY MEN

Previous studies have demonstrated that expression of FGF21 is inducible by fasting in rodents (5, 6). We therefore investigated the effect of fasting on serum FGF21 concentrations in humans using the CLIA established above. In the first set of the experiments, 7 healthy male volunteers (from 26 to 47 years old) received a standardized meal at 1700 and then fasted for 24 h. The blood samples were taken at 1800 on the same day and at 0900 and 1800 on the second day. Repeated-measures ANOVA demonstrated that the concentrations of FGF21 changed significantly over time (P < 0.001). Post hoc paired sample t-tests were then used to compare concentrations between pairs of time points. After Bonferroni correction, the serum concentrations of FGF21 were found significantly increased after overnight fasting in the 7 study participants (P < 0.001) (Fig. 2A). Unexpectedly, prolonged fasting for 24 h did not cause further increases in serum FGF21. Instead, serum FGF21 concentrations at 1800 markedly decreased to a level comparable to that at 1800 of day 1 (P < 0.001).

To closely monitor the circadian changes in serum FGF21, blood samples were taken every 30 min from another 10 healthy volunteers in the fasting condition. This analysis showed an oscillatory pattern of serum FGF21 over 24 h (Fig. 2B). The biggest increase of serum FGF21 concentrations started around midnight (0000), reached its peak at around 0500 of the second day, and then slowly declined to a nadir in the afternoon, suggesting that FGF21 secretion is regulated by the diurnal rhythm in humans. Consistent with the findings in humans, serum FGF21 concentrations in C57 mice also displayed a 24-h circadian rhythm under fasting conditions (see Fig. 1 in the online Data Supplement). The peak concentration of serum FGF21 in mice was observed at 0400 and then progressively declined.

CIRCADIAN CHANGES OF SERUM FGF21 WITH STANDARDIZED MEALS

To investigate whether the 24-h profile of serum FGF21 is influenced by food intake, we next conducted 2 independent experiments in different cohorts. In the first pilot study, 7 healthy volunteers described in Fig. 2A received a standardized breakfast at 0730 after fasting overnight, and blood samples were taken at 0900 (90 min after food intake). There were no significant changes in mean serum FGF21 concentrations in the cohort fed breakfast compared with individuals who were fasted [Fig. 2C, mean (SD): 146.38 (45.95) ng/L vs 151.14 (20.0) ng/L, P > 0.05]. In another set of studies, 19 volunteers, including 12 lean and 7 obese individuals (Table 1 in the online Data Supplement), were provided with a standardized breakfast, lunch, and dinner at 0800, 1300, and 1800, respectively, and their blood samples were collected every 30 min for a period of 24 h from 0730 at day 1 to 0730 at day 2. The nocturnal rise of serum FGF21 in these individuals was similar to that observed under fasting conditions (Fig. 3A and Fig. 2 in the online Data Supplement). On the other hand, another obvious increase of serum FGF21 was observed in the afternoon. FGF21 started to rise at 1430, peaked at 1630, and then decreased to baseline at 1900. Further analysis showed that baseline serum FGF21 concentrations in obese individuals were higher than those in lean individuals. The nadir serum FGF21 (at 1430) was higher in the obese group [146.38 (18) ng/L vs 73.10 (11.90) ng/L in the lean group, P < 0.05; Fig. 3B], but no
A significant difference was found in FGF21 concentrations at the peak (0500) between the 2 groups. In addition to these indirect measures of diurnal variation, the circadian rhythm of serum FGF21 in both lean and obese individuals could be described by a cosine curve (Fig. 3A, \( R^2 \) lean = 0.656; \( R^2 \) obese = 0.358; both \( P < 0.001 \)). In both groups, the curves had an amplitude significantly greater than zero. The cosine fit of the 24-h FGF21 profile had a similar time course in all the study participants. There was no significant difference in the mean (95% CI) acrophase between the 2 groups [0521 (0456 – 0545) in lean vs 0509 (0438 – 0541) in obese]. The mean (95% CI) amplitudes in the lean group were significantly higher than in the obese group [66.4 (54.7–78.0) vs 49.8 (33.1–66.5) ng/L; \( P < 0.01 \)]. The mean (95% CI) MESOR in the obese group was much higher than that in the lean group [210.4 (202.4–219.1) vs 140.4 (135.1–146.8) ng/L; \( P < 0.01 \)].

When the FGF21 concentrations were expressed as fold changes over the nadir level at 1430, the amplitude of 24-h oscillation in the obese group was not as obvious as that in lean controls (Fig. 3C). The mean (SD) area under curve over 24 h in the obese group was significantly lower than that in the lean group [69.03 (13.68) vs 106.84 (12.9); \( P < 0.01 \)]. The mean (SD) peak/nadir ratio of serum FGF21 concentrations in obese individuals was significantly smaller than that in lean individuals [Fig. 3D, 1.99 (0.31) vs 4.26 (0.47); \( P < 0.05 \)], indicating that the nocturnal rise of serum FGF21 was significantly blunted in the obese group.

**TEMPORAL ASSOCIATION OF FGF21 WITH INSULIN, GLUCOSE, AND CORTISOL**

To further delineate the temporal association of serum FGF21 with other metabolic hormones and metabolites, we analyzed the 24-h profiles of insulin, glucose,

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**Fig. 3.** The oscillatory pattern of serum FGF21 over 24 h with consumption of standardized meals. Blood samples were drawn every 30 min from 19 volunteers (12 lean and 7 obese individuals) who received the 3 standardized meals.

(A), 24-h excursion of serum FGF21 concentrations in lean (n = 12) and obese (n = 7) individuals and their cosine fit curves. (B), FGF21 levels at the nadir (1430) and peak (0500) in lean and obese individuals (**\( P < 0.01 \) compared with lean individuals at 1430). (C), Relative levels of serum FGF21 as expressed as fold-change over the nadir levels (at 1430) in lean and overweight individuals. (D), The ratio of the peak/nadir serum FGF21 in lean and obese individuals (**\( P < 0.01 \) ).
and cortisol in our studies of the 19 individuals. As expected, the serum concentrations of both insulin and glucose displayed characteristic pulsatile increases after the 3 meals (Fig. 4, A and B). The peak concentrations of both insulin and glucose were observed within 30–60 min after each meal. Interestingly, the 24-h excursion of both insulin and glucose tended to be opposite to that of circulating FGF21. During the daytime, the peak time of both serum insulin and glucose after lunch and dinner matched the nadir time of serum FGF21. The lowest concentrations of both insulin and glucose were detected between midnight and early morning when the major nocturnal rise of serum FGF21 occurred. By contrast, the pattern of the nocturnal rise in serum cortisol showed a striking similarity to that of serum FGF21 (Fig. 4C). Both FGF21 and cortisol started to increase at around midnight and peaked at around 0500, followed by a gradual decline.

**ROLE OF FFAs IN REGULATING CIRCADIAN CHANGES OF FGF21**

FFAs have been shown to be a positive regulator of FGF21 production through the activation of PPARα (17). We next analyzed the temporal association of serum FFAs with circulating FGF21 in the 19 volunteers as described above. Consistent with previous reports (22), serum FFA profiles also showed an oscillatory pattern over 24 h, with 2 obvious peaks being detected during the daytime and nighttime, respectively (Fig. 5A). Similar to circulating FGF21, the major rise of FFAs also occurred during the night, with the increase

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**Fig. 4. Temporal association of serum insulin, glucose, and cortisol with FGF21 over 24 h.**
The serum samples were collected from 19 volunteers, as in Fig. 2. Serum levels of insulin (A), glucose (B), and cortisol (C) were measured every 30 min and compared with the pattern of circulating FGF21.

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![Graphs showing temporal association of serum insulin, glucose, and cortisol with FGF21 over 24 h.](image)
starting around 2100, peaking around 0130, and then gradually decreasing to the nadir level at around 1030. Another relatively smaller and transient peak of serum FFAs was observed between 1030 and 1430. Interestingly, this 24-h temporal pattern of serum FFAs was quite similar to that of circulating FGF21, except that the peak time of serum FFAs occurred approximately 4 h earlier than that of circulating FGF21, both during the day and at night. Furthermore, the peak concentrations of serum FFAs correlated significantly with the peak concentrations of circulating FGF21 during both the day and the night (Fig. 5, B and C). A significant positive correlation between circulating FGF21 and serum FFAs was also observed when the 2 major peaks of FFAs and FGF21 were expressed as area under the curve (Fig. 5, D and E).

To further confirm the above in vivo findings, we next tested the direct effects of different types of FFAs on FGF21 mRNA expression in human HepG2 hepatocytes. Treatment of cells with linoleate (Fig. 6) or oleate (data not shown) increased FGF21 mRNA expression and its protein secretion in a time- and concentration-dependent manner. The maximal increase of FGF21 mRNA expression was observed at approximately 4 h after treatment with linoleate, which matched well with the time lag of the peak concentra-

**Fig. 5.** The close correlation between the circadian changes of serum FFAs and FGF21. Serum samples were collected, as in Fig. 2. (A), Comparison of 24-h profiles of serum FFAs and FGF21. (B and C), Correlation between the peak levels of serum FGF21 and FFAs during the day (B) and night (C). (D and E), Correlation between the peak serum FGF21 and FFAs calculated as the area under curve (AUC) during the day (D) and night (E). AUC was calculated using the trapezoidal rule.
tions between FFAs and FGF21 observed during both daytime and nighttime. On the other hand, palmitate did not have any obvious effect on FGF21 mRNA expression or protein secretion.

Discussion

The secretion of many metabolic hormones is characterized by biological rhythms that may reflect their physiological roles in metabolic regulation. The present study provides the first evidence showing that circulating FGF21 concentrations exhibit a characteristic diurnal rhythm in humans, with a major nocturnal rise occurring between midnight and early morning. These findings are in keeping with the hypothesis that FGF21 may be a metabolic regulator in humans.

A number of metabolic hormones, including leptin (23), melatonin (24), growth hormone (25), vaspin (26), and cortisol (27), have been shown to exhibit a nocturnal rise, constituting an important mechanism for metabolic adaptation. For example, the nocturnal rise of leptin might be related to its appetite-suppressing effect during nighttime sleep (23). The present study demonstrates that the nocturnal rise of FGF21 closely resembles that of cortisol, which is secreted from the adrenal gland in circadian cycles (25). Interestingly, both the onset and peak time of the nocturnal rise are almost the same for FGF21 and cortisol, suggesting that these 2 hormones might be controlled by a similar set of circadian clock genes. The nocturnal rise of cortisol has been suggested to play a key role in enhancing hepatic glucose production during the night (27). Likewise, FGF21 also stimulates hepatic gluconeogenesis through induction of PGC1α in mice (8), suggesting that the nocturnal rise of FGF21 may be actively involved in maintaining glucose homeostasis during the night.

Fig. 6. Effects of fatty acids on FGF21 mRNA expression and protein secretion in hepatocytes.

Human HepG2 cells grown under serum-free conditions were treated with linoleate (200 μmol/L) and palmitate (200 μmol/L) or vehicle control (1% BSA) for various time periods. (A), The relative mRNA levels of FGF21 as determined by quantitative real-time PCR (expressed as fold-changes over vehicle control in each time point). (B), Protein levels of FGF21 secreted into conditioned medium. FGF21 protein concentrations in the supernatant were measured with immunonasay, normalized against total protein, and expressed as fold-change over vehicle control in each time point. Changes in relative FGF21mRNA levels (C) and protein secretion (D) after treatment of HepG2 cells with different concentrations of linoleate and palmitate for 24 h were analyzed, as in (A) and (B), respectively. *P < 0.05; **P < 0.01 vs control group (n = 5–6).
Previous studies in different ethnic groups have consistently demonstrated increased concentrations of circulating FGF21 in obese individuals \(11, 28\). In line with these reports, the present study also showed higher circulating FGF21 in obese individuals during the daytime compared with healthy controls. However, obese individuals displayed an impaired nocturnal rise and reduced circadian rhythmicity in circulating FGF21. Noticeably, disrupted circadian periodicity of several metabolic hormones has been implicated in the development of obesity and its related disorders. For instance, the diminution of nocturnal release in both growth hormone and melatonin may result in increased body weight, visceral obesity, and adverse metabolic consequences \(24, 25\). By contrast, restoration of the nocturnal melatonin signal reduces body weight and abdominal adiposity, leading to improved insulin sensitivity \(29\). In this connection, our findings raise the possibility that the diminished circadian rhythms of FGF21 in obese individuals may contribute to obesity-mediated metabolic dysregulation. It is likely that the sustained increase in circulating FGF21 during the daytime may cause desensitization or resistance of this hormone, thereby dampening the metabolic effects of the nocturnal rise in FGF21 in obese individuals.

Our present findings in humans are also supported by data from both animal and in vitro studies. In mice, the PPAR\(\gamma\) agonist bezafibrate induces FGF21 expression in a circadian manner \(30\). Our 24-h profiling analysis of circulating FGF21 in fasting mice also shows a circadian pattern of FGF21 reminiscent of that in humans. In cultured cells, hepatic expression of FGF21 is regulated by several factors that are the key components of the circadian clock, including retinoid acid receptor–related orphan receptor \(\alpha\) (RO\(\alpha\)) \(31\), PGC1\(\alpha\), and Rev-Erb\(\alpha\) \(32\). Conversely, FGF21 has been reported to induce the expression of the circadian clock gene peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC-1\(\alpha\)) also known as PPAR\(\alpha\) \(8\), suggesting the existence of a feedback regulation between FGF21 and the circadian clock system. Taken in conjunction, these findings highlight the role of FGF21 as an integral component of the circadian clock genes in humans.

Fatty acids have been shown to be a positive regulator of hepatic FGF21 production through the activation of PPAR\(\alpha\) \(17\). The present study provides several lines of evidence, suggesting that the circadian rhythm of FGF21 is controlled at least in part by the oscillatory pattern of circulating FFAs. First, the 24-h profiles of circulating FFAs closely resemble those of FGF21. Second, there is a strong positive association between the peak concentrations of circulating FFAs and FGF21 both during the day and at night. Third, the peak time of circulating FFAs precedes that of FGF21 by approximately 3–4 h, matching well with our in vitro observation that incubation of human hepatocytes with fatty acids for this period induced the production of FGF21. Notably, both our study and a previous report \(17\) showed that polyunsaturated, but not saturated, fatty acids, induce hepatic FGF21 expression. The mechanisms underlying the differential effects of these 2 types of fatty acids remain to be established. Because PPAR\(\alpha\) plays an indispensable role in fatty acid–induced FGF21 expression \(17\), it is possible that polyunsaturated fatty acids are more potent in activation of PPAR\(\alpha\).

Interestingly, FGF21 regulates the production of FFAs through the modulation of lipolysis in white adipocytes \(6, 9, 33\). A recent study in FGF21 knockout mice showed that FGF21 inhibits lipolysis and decreases FFAs under fasting conditions \(9\). Taken together, these findings suggest the existence of a feedback regulation between FFAs and FGF21. It is likely that the nocturnal rise of FFAs leads to a subsequent increase in circulating FGF21, which in turn decreases FFAs through the inhibition of lipolysis in white adipocytes. In summary, the results of the present study demonstrate that the circadian rhythm of FGF21 is closely related to the diurnal changes in fatty acids in humans and also suggests that the dysregulated circadian rhythmicity in FGF21 may be causally associated with obesity-related lipid abnormality and insulin resistance. The reciprocal regulation between FGF21 and FFAs may play an important role in mediating the cross-talk between adipose tissue and liver and warrants further investigation.

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