Preserved Circadian Rhythm of Serum Insulin Concentration at Low Plasma Glucose During Fasting in Lean and Overweight Humans

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Circadian rhythms in glucose metabolism are well documented. Most studies, however, evaluated such variations under conditions of continuous glucose supply, either via food intake or glucose infusion. Here we assessed in 30 subjects circadian variations in concentrations of plasma glucose, serum insulin, and C-peptide during a 72-hour fasting period to evaluate rhythms independent from glucose supply. Furthermore we assessed differences in these parameters between normal-weight (n = 20) and overweight (n = 10) subjects. Blood was sampled every 4 hours. During fasting, plasma glucose, serum insulin, and C-peptide levels gradually decreased (all $P < .001$). While there was no circadian variation in plasma glucose levels after the first day of fasting, serum levels of insulin were constantly higher in the morning (8.00h) than at night (0.00h) ($P < .001$), although the extent of this morning-associated rise in insulin levels decreased with the time spent fasting ($P = .001$). Also, morning C-peptide concentrations were higher compared to the preceding night ($P < .001$). The C-peptide/insulin ratio (CIR) decreased during prolonged fasting ($P = .030$), suggesting a decrease in hepatic insulin clearance. Moreover, CIR was significantly lower in the morning than at the night of day 1 and day 2 of fasting ($P = .010$ and $P = .004$, respectively). Compared to normal-weight subjects, overweight subjects had higher plasma glucose, as well as serum insulin and C-peptide levels (all $P < .03$). Data indicate preserved circadian rhythms in insulin concentrations in the presence of substantially decreased glucose levels in normal-weight and overweight subjects. This finding suggests a central nervous system contribution to the regulation of insulin secretion independent of plasma glucose levels.

CIRCADIAN variations in glucose metabolism have been extensively studied. Systematic diurnal variations were revealed for blood glucose concentration,[1-3] insulin secretion,[2,4-7] insulin sensitivity,[8] and glucose tolerance.[9] It was suggested that these variations originate from rhythms of neuroendocrine systems, such as the hypothalamus-pituitary-adrenal (HPA) axis[10] or the sympathoadrenal system,[11] the activity of which is closely linked to central nervous system circadian oscillators, and which simultaneously exert a strong regulatory effect on carbohydrate metabolism.[6,8,12] Notably however, most of those studies evaluating circadian variations in glucose metabolism were conducted while supply of glucose was continued either via food intake[1-3,5,7] or via glucose infusion.[3,4,6,8,9] On this background, it is difficult to dissociate the influences of exogenous glucose supply from intrinsically generated circadian variations in carbohydrate metabolism and insulin secretion. In this context, only a few studies have concentrated on periods of prolonged fasting, which appears to be an advantageous approach for studying spontaneous variations during the 24-hour cycle.[13-17] In some of these studies the fasting period was rather short (≤30 hours)[14,15,17] or blood samples were collected only at a few (1 or 2) time points per day,[13,16] so that the results were not fully conclusive. The most comprehensive study on this issue showed differences between morning and evening concentrations of insulin with distinctly higher levels in the morning even after 72 hours of fasting in 11 normal-weight men.[13] Here, we report effects on concentrations of plasma glucose as well as serum insulin and C-peptide in blood collected every 4 hours during prolonged (72 hours) fasting in 30 subjects who were normal weight and overweight. Also, differences between men and women were assessed.

MATERIALS AND METHODS

Subjects and Fasting Procedure

The fasting experiments were performed in 37 subjects who were hospitalized for diagnosis of suspected insulinoma due to episodes of loss of consciousness (12 subjects) or of other symptoms suspect for hypoglycemia, eg, nervousness, palpitation, and tremor (25 subjects). In 3 of these subjects diagnosis of insulinoma was confirmed (by plasma glucose levels < 2.2 mmol/L after starvation) and were excluded. Four further subjects were excluded from analysis, since they did not adhere to the instruction of fasting.

The underlying cause for the symptoms experienced by the subjects for whom diagnosis of insulinoma was excluded remained obscure in the 30 subjects included here, despite careful examination. All subjects who had experienced an episode of loss of consciousness (9 subjects in the final sample) had a computed tomography (CT) scan of the cranium, a duplex-sonogram of the brain arteries, an orthostatic standing test, a long-term electrocardiogram registration, and an echocardiogram. Based on the result of the orthostatic standing test, 7 of these subjects were assumed to have had an orthostatic syncope, but this could not be further proven. After exclusion of insulinoma and 3 days of observation in the hospital for most subjects, psychosomatic contributions were suspected and a psychologic evaluation was recommended. However, the patients were not followed up, so that the accuracy of this diagnosis was not determined. It should be pointed out that none of the subjects included in the final sample had diabetes mellitus (morning fasting glucose concentration < 7 mmol/L).

After consuming a normal breakfast in the morning of the first day, patients started to fast. The first blood sample was collected at noon of the first day; further samples were taken every 4 hours until 8:00h of the fourth day. Patients were allowed to drink water and unsweetened tea. They could walk around in the hospital area and had regular sleep times, except for minor disturbances due to nocturnal blood sampling.

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Table 1. Characteristics of the Subjects' Subgroups

	Men (n = 10)	Women (n = 20)	Normal Weight (n = 20)	Overweight (n = 10)
Male/female	—	—	6/14	4/6
BMI (kg/m^2)	28.4 ± 2.4	24.8 ± 1.0	22.7 ± 0.5	32.6 ± 1.7
Age (yr)	45.7 ± 5.1	36.1 ± 3.4	41.5 ± 3.9	35.1 ± 4.1

NOTE. Values are means ± SEM.

Plasma glucose concentration was measured by glucose hexokinase method. Serum insulin and C-peptide concentrations were assessed using enzyme immunoassays (DAKO Cytomation, Cambridgeshire, UK; insulin: interassay coefficient of variation [CV] 7.5%, intra-assay CV 6.7%; C-peptide: interassay CV 5.2%, intra-assay CV 4.7%).

Statistical Analysis

All data are presented as means ± SEM. To estimate the hepatic insulin clearance and insulin requirements for glucose homeostasis, the C-peptide/insulin ratio (CIR) and the insulin/glucose ratio (IGR) were calculated, respectively.[18-21] Repeated-measures analyses of variance (ANOVAs) were performed including the factors "day" (1st, 2nd, 3rd) and "time" (12:00h, 16:00h, 20:00h, 0:00h, 4:00h, 8:00h) to assess temporal changes in the different parameters. Gradual changes during fasting were thus expressed as the main effect of "day" and systematic diurnal variations as main effect of "time". In addition, differences between nighttime (0:00h or 4:00h) and morning concentrations (8:00h) were assessed using Student's *t* test separately for each day of fasting. To evaluate modulating influences of body weight and gender on changes in variables during fasting, the subjects were subdivided according to, respectively, gender and body mass index (BMI) with a BMI ≥27.0 kg/m^2 defining overweight and a BMI < 27.0 kg/m^2 normal weight. Subsequently, the factors "sex" and "BMI" were included into the ANOVA models. A P value <.05 was considered significant.

RESULTS

The characteristics of the subject sample are shown in Table 1. During the 72-hour fasting period, plasma glucose dropped on average from 4.94 ± 0.11 mmol/L to 3.36 ± 0.13 mmol/L ($P < .001$ for "day", Fig 1). Also, a significant decrease across days was observed in concentrations of insulin (from 45.4 ± 5.51 pmol/L to 31.6 ± 3.36 pmol/L, $P < .001$) and C-peptide (from 689 ± 67.4 pmol/L to 376 ± 32.1 pmol/L, $P < .001$), as well as for the CIR (from 17.5 ± 1.16 to 13.9 ± 1.52, $P =$

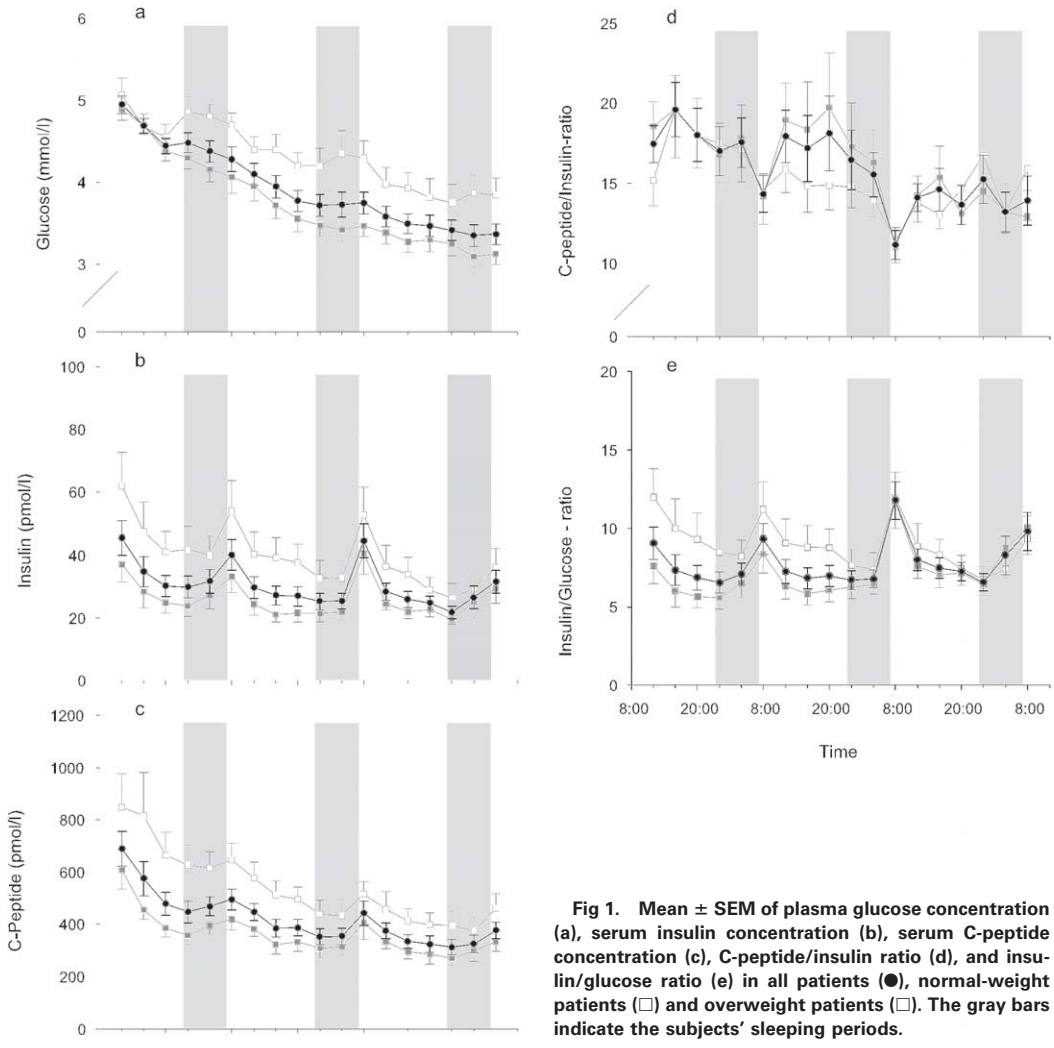

Fig 1. Mean ± SEM of plasma glucose concentration (a), serum insulin concentration (b), serum C-peptide concentration (c), C-peptide/insulin ratio (d), and insulin/glucose ratio (e) in all patients (●), normal-weight patients (□) and overweight patients (□). The gray bars indicate the subjects' sleeping periods.

.030). However, IGR showed no significant changes in the course of the experiment (from 9.05 ± 1.02 to 9.79 ± 1.21, $P = .888$).

Glucose concentrations decreased constantly during each day ($P < .001$ for "time"), but except for the first day, no significant differences between nighttime and morning concentrations were found (Table 2). However, the decrease in plasma glucose levels was more pronounced during the first day than during the last day ($P = .043$ for "time" × "day"). Insulin concentrations in the morning were constantly higher than at nighttime ($P < .001$ for "time") and the same was true for C-peptide concentrations ($P < .001$ for "time"). The extent of these circadian rhythms in insulin and C-peptide concentrations decreased during the course of the experiment ($P < .001$ for "time" × "day" for both hormones), but the diurnal variation was still detectable on the last day (Table 2). Of note, the CIR was distinctly lower in the morning than at nighttime on days 1 and 2, but not on day 3 ($P < .001$ for "time", Table 2). IGR, like insulin concentrations, showed a clear peak around 8.00 h ($P < .001$ for "time", Table 2).

Throughout the fasting period, glucose ($P = .009$), insulin ($P = .025$), and C-peptide ($P = .003$) concentrations were significantly higher in overweight than in normal-weight subjects. However, the decrease in plasma glucose concentrations was significantly lower in overweight than in normal-weight subjects ($P < .001$ for "time" × "BMI":). However, for CIR no differences between overweight and normal-weight subjects were observed ($P > .3$ for all comparisons). The IGR was higher in the overweight than in the lean subjects at the beginning of the fasting period, and showed distinctly different time-courses in the 2 groups, decreasing in overweight and increasing in normal-weight patients, so that values were quite similar at the end of the fast ($P = .006$ for "day" × "BMI").

Analyses of gender effects showed lower plasma glucose concentrations in women than men ($P = .020$ for "sex", Fig 2), which might be related to the slightly lower BMI in the women than the men (24.8 ± 1.0 kg/m^2 *v* 28.4 ± 2.4 kg/m^2, $P = .113$).

Table 2. Comparison Between Nighttime (0:00h and 4:00h) Nadir Value and Morning (8:00h) Maximum

	Day No.	Nighttime	Morning	*P* Value
Glucose	1	4.48 ± 0.12	4.28 ± 0.15	**.024**
	2	3.72 ± 0.13	3.74 ± 0.13	.747
	3	3.41 ± 0.13	3.36 ± 0.13	.506
Insulin	1	29.8 ± 3.5	40.1 ± 4.9	**<.001**
	2	25.2 ± 2.7	44.6 ± 5.3	**.001**
	3	21.8 ± 1.9	31.5 ± 3.7	**.004**
C-peptide	1	447 ± 42	495 ± 40	**.038**
	2	352 ± 32	442 ± 45	.056
	3	312 ± 29	376 ± 32	**.001**
CIR	1	17.5 ± 1.5	14.3 ± 1.2	**.010**
	2	16.5 ± 1.9	11.2 ± 0.9	**.004**
	3	15.2 ± 1.5	13.9 ± 1.5	.391
IGR	1	6.54 ± 0.68	9.30 ± 1.00	**<.001**
	2	6.71 ± 0.59	11.75 ± 1.22	**<.001**
	3	6.56 ± 0.54	9.79 ± 1.21	**.002**

NOTE. For CIR, the relation is reversed with the maximum at night and nadir values in the morning. Values are means ± SEM values and significance (*P* value printed in bold) for the difference is indicated.

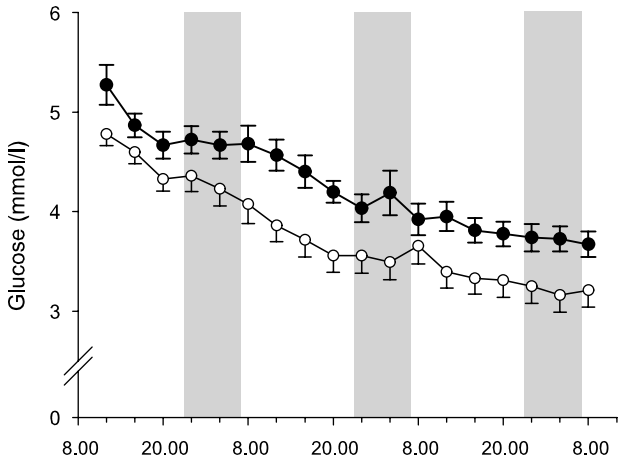

Fig 2. Mean ± SEM of plasma glucose concentration in men (●) and women (○). The gray bars indicate the subjects' sleeping periods.

Including the "sex" factor into analyses of the insulin, C-peptide, CIR, and IGR data essentially did not change results, with the respective interaction terms not reaching significance (all $P > .25$, except for IGR where the "day" × "sex" interaction showed a trend of $P = .068$ with decreasing values in women and stable values in men).

DISCUSSION

Our data show that during prolonged fasting, plasma glucose concentrations decrease gradually without any substantial circadian variations. In contrast, and also in line with previous observations,[13] persistent circadian variations were found during the fast for insulin secretion as estimated by C-peptide and insulin concentrations. This rhythm was characterized by a nadir during the night and maximum concentrations around 8.00h in the morning. Furthermore, this circadian rhythm in insulin secretion was found to be preserved also in overweight subjects, who showed distinctly higher levels of plasma glucose, serum insulin, and C-peptide.

Since blood was sampled only every 4 hours, a rise in plasma glucose concentration that preceded and promoted the increase in morning insulin concentration could have been missed in the present study. Activity of counterregulatory endocrine systems, like the sympathetic nervous system (SNS) and the HPA axis are known to reach a maximum around awaking in the morning hours, which might have temporally increased plasma glucose levels and decreased insulin sensitivity, often referred to as the "dawn phenomenon."[10,11] However, during prolonged fasting we would expect the increases in plasma glucose levels in the morning to be quite small, not inducing substantial insulin secretion.[22] Thus, the finding of a preserved circadian variation in plasma insulin levels in the presence of generally low plasma glucose levels during fasting appears to be a surprising one, the origin of which remains to be elucidated.

In principle, 2 mechanisms could mediate the morning rise in insulin concentrations. First, secretion of insulin from the pancreas could be enhanced and, second, hepatic clearance of insulin could be diminished. However, the view of an increased

hepatic clearance is not supported by the results regarding C-peptide, which is secreted together with insulin from the pancreas in equimolar amounts but is not substantially extracted by the liver.[20,21,23] In parallel with insulin, concentrations of C-peptide showed a clear maximum in the morning, suggesting that these peaks were induced by increased secretion rather than decreased hepatic clearance. On the other hand, the CIR, which has been considered a reliable measure of hepatic insulin extraction,[20,21] dropped in the morning of days 1 and 2. This drop may be taken to argue that a decrease in hepatic insulin clearance has partly contributed to the morning rise in insulin.

Throughout the fasting period the overweight subjects had higher levels of glucose and insulin than the lean subjects, indicating a state of relatively enhanced insulin resistance that has been consistently observed in overweight subjects.[16] On this background, it is interesting that the circadian pattern of insulin secretion was preserved in our overweight subjects, despite those obvious deviations in glucose metabolism. Taken together, the normal circadian variation in serum insulin concentrations in conjunction with higher absolute levels of circulating insulin and glucose suggests an elevation of the set point in glucose regulation in obesity, while the circadian regulation per se appears to be intact. Whether this alteration in glucose metabolism contributes to the development of obesity, or vice versa is a consequence of the diseases, cannot be answered on the basis of the present data.

In addition to the higher plasma glucose levels in women than men, which cannot be fully explained here, analyses did not reveal any substantial dependence of circadian changes in the variables of interest on gender, suggesting that the observed variations during fasting pertain to both sexes. However, since only a small number of subjects were studied, statistical power was low and more discrete differences in the regulation of glucose metabolism and insulin secretion during fasting between sexes may have been missed.

In a previous study[22] endogenous insulin secretion was shown to be abolished during acute insulin-induced hypoglycemia. This suppression of insulin secretion by low plasma glucose levels did not depend on the dose of insulin infused. This maximum suppression of insulin secretion was reached already at mild levels of hypoglycemia (~3.5 mmol/L). Here, despite the fact that prolonged fasting lowered plasma glucose concentrations to a comparable level, insulin secretion was not completely suppressed. Thus, acutely induced hypoglycemia and developing hypoglycemia during starvation represent 2 different metabolic states with differing effects on insulin secretion.

CIR has previously been found to represent quite well the hepatic clearance of insulin.[20] In this study, CIR was distinctly decreased in the morning of days 1 and 2. Other studies employing different measures of hepatic insulin clearance revealed inconsistent results on circadian variations. While 1 study[24] showed a decrease in insulin clearance in the morning similar to the present results, others showed increases[25,26] or no changes at this time of day.[27,28] Since none of the previous studies examined insulin clearance during fasting but during ongoing glucose supply, it can be questioned whether the results are comparable with those of the present study. Although the present data should be interpreted with caution since CIR probably does not represent the most accurate method to measure hepatic insulin clearance, they may be taken as a first sign that hepatic clearance of insulin is subject to a circadian rhythm, a subject worth further investigation.

It should be pointed out that data in the present study were collected in subjects who had unexplained symptoms compatible with those typically experienced during hypoglycemia. Hypoglycemia (plasma glucose < 2.6 mmol/L) did not occur in any of the subjects during the 72-hour fasting period and, thus, the presence of insulinoma can be excluded. Nevertheless, most subjects were not completely healthy but suffered from an undefined disorder, probably of psychosomatic type. On this background, we cannot exclude that these disorders in some cases have confounded the present results, which, therefore, can be generalized to a normal population only with caution. However, the fact that none of the subjects exhibited any clear-cut disturbance in glucose regulation renders a systematic influence of nonmetabolic disorders on our results unlikely.

In conclusion, this study indicates that the circadian rhythm of circulating insulin is preserved during a prolonged fast in normal-weight as well as in overweight humans, despite low plasma glucose concentration. The finding suggests that during fasting, insulin secretion does not solely depend on plasma glucose concentration but rather on other neuroendocrine factors regulated via central nervous system circadian oscillators.

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