Differences Between Nighttime and Daytime Hypoglycemia Counterregulation in Healthy Humans

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Disturbances in hormonal counterregulation may be the main reason why many type 1 diabetic patients are asymptomatic during nighttime hypoglycemia. While it is known that sleep attenuates counterregulatory responses to hypoglycemia, the influence of the time of day on hormonal counterregulation regulation remains obscure. We induced hypoglycemia at 2 different time intervals, ie, in the morning and in the early night, in healthy subjects staying awake throughout the experiments. As compared with the morning hypoglycemia, epinephrine response during early nighttime hypoglycemia was markedly enhanced ($P < .001$). Baseline corticotropin (ACTH) and cortisol levels were higher in the morning than during nighttime ($P < .001$ for both). However, the increase of both hormones was stronger at nighttime ($P = .045$ and $P < .001$, respectively), so that at the end of the hypoglycemic clamp, levels at nighttime were comparable to morning levels. In the morning, the increase in glucagon levels was more pronounced than during nighttime ($P = .019$), but given that baseline glucagon levels were distinctly higher at nighttime than in the morning ($P = .003$), at the end of the clamps, levels of this hormone remained still higher at nighttime than in the morning ($P = .017$). The increase in growth hormone during hypoglycemia did not differ between morning and nighttime ($P = .728$). Data shows that several components of hormonal counterregulation against hypoglycemia are influenced by the time of day. Especially, the markedly enhanced epinephrine response to early nighttime hypoglycemia could be clinically important, because this neuroendocrine response is known to play a crucial role in mediating the awareness of and metabolic defensive mechanism against hypoglycemia.

HYPOGLYCEMIA IS THE limiting factor of insulin therapy in type 1 diabetes (T1DM).[1] During nighttime sleep, hypoglycemia represents a particular problem, since 27% to 56% of T1DM patients during hospitalization have been found to exhibit hypoglycemias with durations between 2 to 12 hours.[2-6] The frequency of nocturnal hypoglycemic episodes in these patients during everyday life is probably similar, although most of the episodes are not recognized, due to the patients' failure to wake up in response to hypoglycemia and the lack of routine blood glucose measurements during nighttime.[7] One explanation for the high frequency of nocturnal hypoglycemia derives from observations by Jones et al,[8] that sleep markedly attenuates hormonal counterregulatory responses to hypoglycemia. Extending this finding, we recently showed that sleep increases the glycemic thresholds for neuroendocrine counterregulatory activation (ie, starting at lower plasma glucose levels during sleep than wakefulness), but does not completely suppress counterregulatory responses.[9] While these studies demonstrated a role of sleep for hypoglycemia counterregulation, it remained still unclear whether the counterregulatory response also depends on the time of day at which hypoglycemia takes place, ie, on circadian influences. Considering that the secretion of many counterregulation hormones, such as epinephrine[10] and the hormones of the hypothalamic-pituitary-adrenal (HPA) axis,[11] exhibit a characteristic circadian secretion pattern, it appears highly likely that hypoglycemic counterregulation may likewise be subject to influences of time of day.

A most comprehensive study dissecting influences of time of day and sleep on hypoglycemic counterregulation has been published recently by Cryer's group.[12] Stepwise hypoglycemic clamp experiments performed in waking subjects either in the morning (7:30 AM to 12:30 PM) or at night (9 PM to 2 AM) did not reveal differential counterregulatory responses, which lead the investigators to exclude the presence of distinct diurnal variation in the physiologic responses to hypoglycemia. However, the clamps of that study lasted 5 hours (300 minutes) with blood samples collected only every 30 minutes. Thus, compared with the present study with clamps lasting only 75 minutes and blood samples being collected every 15 minutes, rather long time periods than different short time intervals during the 24-hour day cycle were studied. It could well be that with such extended periods of hypoglycemia, discrete diurnal variations between different time points were masked. Also, prolonged hypoglycemia may represent a too strong stimulus to discover discrete diurnal variations.

To address this issue and to further elucidate the influence of time of day, we performed single step hypoglycemic clamps lasting only 75 minutes in the morning and during the early night. Experiments were performed in healthy subjects, because in T1DM patients counterregulatory responses to hypoglycemia are likely to show a great variability due to influencing factors, such as antecedent hypoglycemic episodes[13,14] or autonomic neuropathy,[15] which are difficult to control for and which may mask time of day effects. Also, it is well documented that counterregulatory responses to hypoglycemia significantly differ between men and women.[16] Given this background, the study included only healthy men to minimize variability of responses due to other factors, eg, gender or diabetes.

SUBJECTS AND METHODS

Thirty young, healthy men participated in the experiments. Exclusion criteria were chronic or acute illness, current medication of any kind, smoking, alcohol or drug abuse, obesity, diabetes, and hyperten-

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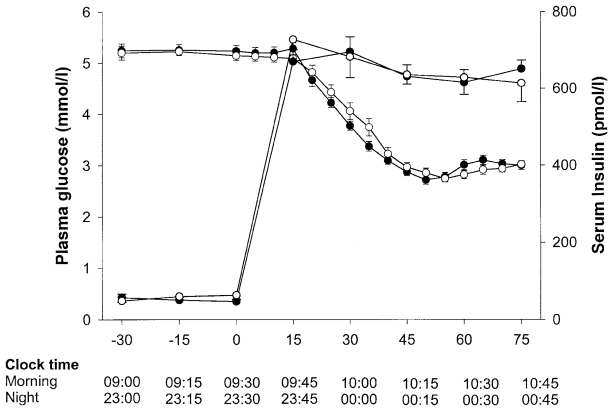

Fig 1. Mean ± SEM plasma glucose concentrations (mmol/L) and serum insulin concentrations (pmol/L) during the nighttime (11 PM to 0045h, ●) and daytime (9 AM to 10:45 AM, ○) hypoglycemic clamp. Time axis: −30 to 0 minute: baseline, 0 to 75 minutes: clamp experiment.

sion. All subjects had a regular sleep-wake-cycle during the 4 weeks before the experiments and had not worked on night shifts. Each volunteer gave written informed consent and the study was approved by the local ethics committee.

The subjects were randomly assigned to 2 different groups of the same size. The 15 subjects in each group were comparable in age (25.7 ± 0.9 years *v* 26.7 ± 0.9 years) and body mass index (BMI) (22.3 ± 0.3 kg/m^2 *v* 23.5 ± 0.5 kg/m^2). One group underwent a nocturnal hypoglycemic clamp, the other group a hypoglycemic clamp in the morning. The subjects were not told whether they underwent a euglycemic or hypoglycemic clamp to exclude an influence on the subject's expectation (ie, to undergo a hypoglycemic clamp) on counterregulatory responses.[17] Therefore, a between-subjects design was used to keep the subjects blind towards the hypoglycemic condition.

Subjects reported to the medical research unit at 8 AM and 8 PM, respectively. The subjects of the morning group were allowed to eat until 10 PM at the preceding day; those of the nighttime group were allowed to eat until 7 PM. All subjects had to abstain from eating until the end of the hypoglycemic clamp. The experiments took place in a sound-attenuated room with the subjects sitting with their trunk in an almost upright supine position (about 60°) and their legs in a horizontal position on a bed.

For the clamp, 2 intravenous cannulae were inserted, 1 into a vein in the back of the hand, 1 into an antecubital vein of the other arm. The hand with the cannula was then placed in a heated box (temperature 50°C to 55°C) to obtain arterialized venous blood. Each cannula was connected to a long thin tube, which enabled blood sampling and adapting the dextrose infusion rate from an adjacent room without being noticed by the subject.

After a baseline-period of 30 minutes, starting respectively at 11 PM and 9 AM, hypoglycemia was induced by infusing insulin (H-insulin; Aventis, Frankfurt, Germany) at a constant rate of 1.5 mU min^{-1} · kg^{-1}. Glucose levels were measured online every 5 minutes, using a glucose oxidase method (Beckman Glucose Analyzer, Munich, Germany). Plasma glucose concentration was allowed to decrease to a nadir of 2.8 mmol/L (50 mg/dL) before a 20% dextrose infusion was started to maintain plasma glucose at this level. After a total of 75 minutes, ie, at 0045h and at 10:45 AM, respectively, insulin infusion was stopped and dextrose infusion continued, until normoglycemia was reached again. Throughout the experimental epoch, blood samples were collected every 15 minutes to measure concentrations of epinephrine, norepinephrine, corticotrophin (ACTH), cortisol, glucagons, and growth hormone in serum and plasma, respectively, using standard procedures as previously described.[18]

All values are presented as mean ± SEM. Statistical analysis was based on analysis of variance (ANOVA) for repeated measures including the factor "hypo" for repeated measurements during the clamp and "time" for differences between the morning and nighttime condition. The interaction term of both factors ('hypo × time') indicated differences in response to hypoglycemia between conditions. Additionally, baseline levels as well as peak levels were compared by unpaired Student's *t* tests. All analyses were performed using SPSS software (version 11.0) (SPSS, Chicago, IL). A *P* value of <.05 was considered statistically significant.

RESULTS

The course of plasma glucose, as well as serum insulin concentrations, was comparable between both conditions throughout the clamps (Fig 1). Hormonal data on baseline and peak concentrations in counterregulatory hormones, in response to hypoglycemia are summarized in Table 1. Baseline plasma epinephrine concentration did not differ between conditions ($P = .127$). In response to hypoglycemia, epinephrine concentrations increased markedly during both conditions, with this increase being distinctly more pronounced during nighttime than in the morning ($P < .001$ for "hypo × time" interaction), so that at the end of the nocturnal clamp, plasma concentrations were nearly twice as high as during the morning clamp ($P < .001$, Fig 2A). Average baseline norepinephrine concentrations were lower during nighttime than during daytime ($P = .023$). The increase of plasma norepinephrine during the clamps ($P = .826$ for "hypo × time" interaction) was similar in both conditions, and there was also no significant difference in concentration at the end of the clamp ($P = .673$, Fig 2B).

As expected, baseline ACTH and cortisol concentrations were higher in the morning than at night ($P < .001$ for both

Table 1. Mean ± SEM Plasma and Serum Concentrations of Counterregulatory Hormones at Baseline as Well as Peak Responses to Hypoglycemia During the Hypoglycemic Clamp ($Peak_{value} - Baseline_{value}$)

	Night	Morning	*P* Value
Epinephrine (pmol/L)			
Baseline	131 ± 21	87 ± 16	.127
Peak	4,274 ± 388	1,981 ± 262	<.001
Norepinephrine (nmol/L)			
Baseline	0.82 ± 0.08	1.1 ± 0.10	.023
Peak	1.63 ± 0.16	1.73 ± 0.17	.673
ACTH (pmol/L)			
Baseline	1.9 ± 0.1	4.6 ± 0.4	<.001
Peak	30.8 ± 4.7	23.3 ± 3.7	.216
Cortisol (nmol/L)			
Baseline	58 ± 22	298 ± 25	<.001
Peak	483 ± 19	510 ± 22	.339
Growth hormone (μg/L)			
Baseline	1.1 ± 0.4	1.2 ± 0.2	.855
Peak	21.0 ± 2.44	18.2 ± 2.6	.449
Glucagon (ng/L)			
Baseline	236 ± 26	131 ± 19	.003
Peak	256 ± 24	177 ± 20	.017

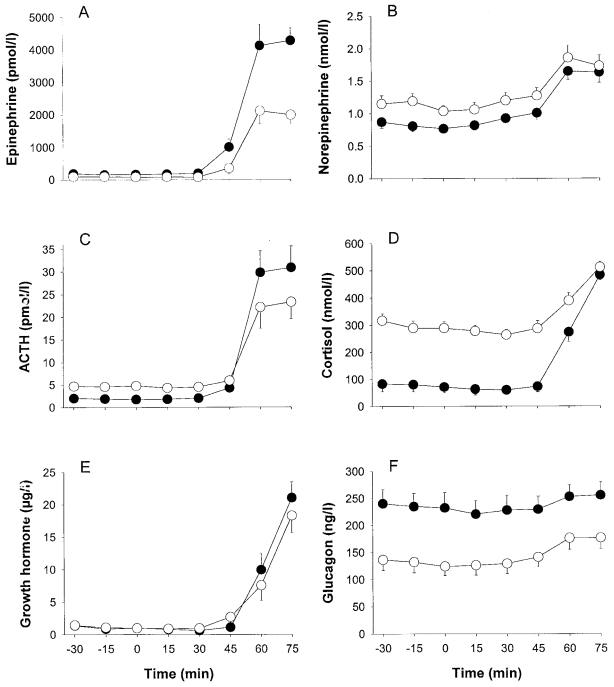

Fig 2. Mean ± SEM serum or plasma concentrations of (A) epinephrine, (B) norepinephrine, (C) ACTH, (D) cortisol, (E) growth hormone, and (F) glucagon, respectively, during the nighttime (11 PM to 0045h, ●) and daytime (9 AM to 10:45 AM, ○) hypoglycemic clamp. Time axis: −30 to 0 minute: baseline, 0 to 75 minutes: clamp experiment.

comparisons). Yet, increases in ACTH and cortisol concentrations were more pronounced during nighttime than in the morning ($P = .045$ and $P < .001$, respectively, for "hypo × time" interaction), so that at the end of the clamp, the ACTH and cortisol concentrations did not differ between both conditions ($P > .2$ for both comparisons, Fig 2C and D).

Growth hormone concentrations increased during both nighttime and morning hypoglycemia without any difference between conditions neither in the increment ($P = .728$ for "hypo × time" interaction) nor in the peak level ($P = .449$, Fig 2E).

Baseline glucagon levels at nighttime were distinctly higher than during the morning ($P = .003$). Despite a more pronounced increase in plasma glucagon upon hypoglycemia in the morning ($P = .019$ for "hypo × time" interaction), concentrations in this condition did not reach the level of nighttime glucagon concentrations (P = .017, Fig 2F).

DISCUSSION

Our data obtained in healthy waking men indicates that several components of hormonal counterregulation against hypoglycemia depend in their amplitude on the time of day. While growth hormone, as well as norepinephrine responses, was found to be similar, the epinephrine response to hypoglycemia was markedly enhanced during the early night as compared with the morning response. Likewise, ACTH and cortisol responses to hypoglycemia were stronger during the early night than in the morning, despite lower baseline concentrations reflecting the well-known circadian nadir of hypothalamic-pituitary-adrenal (HPA)-axis secretory activity during the early night.

Considering the crucial role sympathoadrenal activation plays for the metabolic counterregulation against and for awareness of hypoglycemia in T1DM patients, the most striking finding of the present study is the enhanced epinephrine response to hypoglycemia during the early night.[19] While the finding is in contrast to the results of the above-mentioned recent study,[12] which is most likely explained by differences in study design, it agrees with previous observations of an augmented epinephrine response to nocturnal hypoglycemia compared with diurnal hypoglycemia in awake T1DM patients in a study, which was designed quite similarly to the present study.[8] However, because in that study the focus was on the impact of sleep on hypoglycemic counterregulation, comparisons between daytime and nighttime responses in the waking patients were not performed. An enhanced epinephrine response to nocturnal hypoglycemia as compared with daytime response in T1DM patients was likewise suggested by another study,[20] examining counterregulatory responses after bolus injection of insulin. Lacking a clamp design and control of the hypoglycemic level and duration induced by the insulin injection, the data allow only limited conclusions. On this background, the present data provide clear-cut confirmatory evidence for an enhanced epinephrine response to hypoglycemia during the early night, ie, for an effect of time of day. Of interest, a similar circadian difference in sympathoadrenal activation has also been shown in response to exercise as another stimulus for this neuroendocrine system.[21] However, it remains unclear whether the nocturnal enhancement of the epinephrine response is associated with an increased perception of autonomic symptoms, which was not assessed here and could be of direct clinical relevance.

In contrast to epinephrine, the increase in norepinephrine concentrations during hypoglycemia did not differ between both conditions, which might be explained by the fact, that norepinephrine is to a great extent released directly in the tissue with limited spillover into circulating blood.[22] Thus, plasma norepinephrine levels may not sensitively enough reflect activation of sympathetic nervous system during hypoglycemia to unravel a circadian influence on this system.

While data on the catecholamine response to hypoglycemia are rare, the influence of time of day on the hypoglycemia-induced responses of the HPA-axis and growth hormone has been investigated in several earlier studies.[12,23-25] Although different designs were used and none of the earlier studies employed a glucose clamp technique, their results are remarkably consistent with the present findings, showing greater increment in cortisol levels during nighttime than morning hypoglycemia. Also, those studies did not observe differences in the growth hormone response between nocturnal and morning hypoglycemia.

The greater increase of ACTH and cortisol secretion during nighttime hypoglycemia shown here may provide new insight into the regulation of HPA-axis secretion activity. Considering, that despite different baseline concentrations similar peak concentrations were reached, one may presume that the downregulation of ACTH and cortisol secretion during nighttime is not

mediated by inhibition, but rather by deactivation of the HPA-axis. In contrast to hypoglycemia, in response to exercise, as a different kind of stress, cortisol increases with a comparable amplitude at any time of day, resulting in different peak levels, which parallel the circadian variation of basal HPA-axis secretion activity.[26] Thus, the diurnal variation of the stress-induced activation of the HPA-axis seems to depend on the kind of stressor (eg, hypoglycemia *v* exercise).

The presence of circadian variations in glucagon secretion independently of sleep has rarely been investigated to date. Several studies obtaining effects of sleep and time of day failed to reveal distinct variations in glucagon concentrations throughout the day.[27,28] Diverging from those foregoing observations, here we find a moderate, but significant, difference in the glucagon response to hypoglycemia with greater responses during the morning than in the early night. The increase in glucagon responsiveness was coupled with distinctly lower baseline glucagon levels in the morning compared with nighttime levels. This pattern represents a novel although preliminary finding of a diurnal variation in the secretion of glucagon, which requires further investigation. If sleep per se has a decreasing influence on glucagon, this might explain previous failures to observe this variation.[29]

Two limitations of the study should be pointed out. First, the hypoglycemic plateau of 30 minutes duration was relatively short so that neuroendocrine responses may not have been in steady state. Thus, results may only represent early responses to hypoglycemia while steady state response could still be similar during the 2 different times of day. Second, the time interval between the last meal and the induction of hypoglycemia differed between the 2 experimental conditions, ie, 4 hours in the nocturnal and 10 hours in the morning hypoglycemia condition. On this background, we cannot exclude that differences in neuroendocrine responses to hypoglycemia between the conditions were confounded by potential differences in portal/hepatic glucose values or by hormonal gut factors.

The present results indicating a distinct diurnal variation of neuroendocrine responses to hypoglycemia appear to be in contrast to results of a foregoing study by Cryer's group.[12] However, the designs of that and the present study also markedly differed. For instance, in the study of Cryer et al,[12] hypoglycemic clamps were performed in a stepwise manner over a time period of 5 hours, while in the present study, single-step hypoglycemia of much shorter duration (with the hypoglycemic plateau lasting only 30 minutes). On this background, it might well be that the divergent results of the 2 studies reflect the distinct differences in the temporal dynamics of the hypoglycemic stimulus.

The mechanism underlying the observed enhanced neuroendocrine responses during nighttime remains obscure and cannot be elucidated by the present study. Since glucose metabolism in general is subject to a profound circadian influence,[30,31] probably induced via hypothalamic oscillators, the enhanced neuroendocrine response to hypoglycemia might reflect another facet of this circadian rhythm.

In conclusion, the present study shows distinct differences in counterregulatory responses to morning and early night hypoglycemia in waking healthy men with enhanced responses of epinephrine and HPA-axis during the early night. It should be pointed out that results were obtained in young, healthy men and therefore cannot be extrapolated to other subjects, eg, women. Also, before the clinical impact of these results in regard to diabetes therapy can be estimated, results should be confirmed in patients with T1DM.

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