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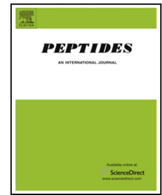

Evidence for time dependent variation of glucagon secretion in mice

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ABSTRACT

Glucose metabolism is subjected to diurnal variation, which might be mediated by alterations in the transcription pattern of clock genes and regulated by hormonal factors, as has been demonstrated for insulin. However, whether also glucagon is involved in the diurnal variation of glucose homeostasis is not known. We therefore examined glucagon secretion after meal ingestion (meal tolerance test) and during hypoglycemia (hyperinsulinemic hypoglycemic clamp at 2.5 mmol/L glucose) and in vitro from isolated islets at ZT3 versus ZT15 in normal C57BL/6J mice and, furthermore, glucose levels and the insulin response to meal ingestion were also examined at these time points in glucagon receptor knockout mice (GCGR−/−) and their wildtype (wt) littermates.

We found in normal mice that whereas the glucagon response to meal ingestion was not different between ZT3 and ZT15, the glucagon response to hypoglycemia was lower at ZT3 than at ZT15 and glucagon secretion from isolated islets was higher at ZT3 than at ZT15. GCGR−/− mice displayed lower basal glucose, a lower insulin response to meal and a higher insulin sensitivity than wt mice at ZT3 but not at ZT15. We conclude that there is a time dependent variation in glucagon secretion in normal mice, which is dependent both on intraislet and extraislet regulatory mechanisms and that the phenotype characteristics of a lower glucose and reduced insulin response to meal in GCGR−/− mice are evident only during the light phase. These findings suggest that glucagon signaling is a plausible contributor to the diurnal variation in glucose homeostasis which may explain that the phenotype of the GCGR−/− mice is dependent on the time of the day when it is examined.

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1. Introduction

Glucose metabolism displays circadian rhythm which is partially a result of dietary intake during the active phase of the 24 h period and maintenance of circulating glucose by hepatic glucose production during the inactive phase [1]. Glucose homeostasis is, however, also regulated by the clock system, both by the clock genes in the suprachiasmatic nuclei in the hypothalamus and by peripheral clock genes in many peripheral organs [1]. Thus, each tissue contains its own circadian clock-program that oscillates over the course of the 24 h day and affects tissue-specific metabolic processes [2,3]. Importantly, unlike the hypothalamus where the main time giver (*zeitgeber*) is the light on the retina [4,5], food intake has been shown to be a stronger *zeitgeber* in peripheral tissues [6]. The intracellular signaling of clock genes consists of interacting transcriptional positive and negative feedback limbs. The negative-feedback limb involves three *Period* genes (*Per1–3*) and two *Cryptochrome* genes (*Cry1* and *2*) in the mouse, whereas the positive-feedback arm involves the genes *Clock* and *Bmal1* [7]. These genes reciprocally regulate each other, establishing an oscillatory pattern of gene transcription.

The importance of the clock genes for glucose homeostasis is evident by findings that genetic deletion of the clock transcription factor in the hypothalamus in mutant mice alters the diurnal feeding pattern and results in overeating, obesity and a sign of metabolic syndrome characterized with hyperglycemia and insulin deficiency [8]. Furthermore, lesion in the suprachiasmatic nuclei disrupts the circadian rhythm of glucose and insulin in mice [9]. Moreover, disruption in the transcription of *Clock* and *Bmal1* alters the expression of genes essential to beta cell function and leads to insulin deficiency and diabetes [10]. The importance of the clock system for glucose homeostasis and islet function is also emphasized by findings that an autonomic rhythm exists within pancreatic beta cells [11,12] and that conditional disruption of the clock in the pancreas results in impaired beta cell function and diabetes [13].

Recently, it was demonstrated that the pancreatic glucagon producing alpha cells is regulated by the clock gene *Rev-erb alpha* such that silencing of this gene inhibits glucagon secretion whereas a *Rev-erb alpha* agonist stimulates glucagon secretion [14]. This would suggest that not only insulin but also glucagon is the subject

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of diurnal variation through clock regulation. This would be of interest since glucagon stimulates hepatic glucose production which is a key mechanism for preventing hypoglycemia during the inactive phase [1]. Interestingly, it has also been reported but not widely discussed, that the phenotype characteristic of a reduction in circulating glucose in glucagon receptor knockout (GCGR−/−) mice is observed only in the morning hours and vanishes later during the day [15], which may further indicate that glucagon is involved in the diurnal variation of glucose homeostasis.

However, besides these studies there is little evidence linking glucagon signaling or glucagon secretion to diurnal variation of glucose homeostasis. To gain further insight in the potential involvement of glucagon in this respect, we have studied a potential time-dependent variation in glucagon secretion in mice by comparing the glucagon response to hypoglycemia and meal test, and glucagon secretion from isolated islets between *zeitgeber* time (ZT) 3 and ZT15 in normal mice and compared glucose levels and insulin response to meal in GCGR−/− mice and their wildtype littermates.

2. Materials and methods

2.1. Animals and anesthesia

Female C57BL/6J mice were obtained from Taconic (Skensved, Denmark) and housed on arrival at 22° in a 12 h light–dark cycle (6 am to 6 pm). The generation of GCGR−/− mice and their wildtype littermates has been described previously [15]. A standard research diet R34 (Lantmännen, Stockholm, Sweden) and water was provided ad lib. Mice were anesthetized prior to all experiments using an intraperitoneal injection of midazolam (18 mg/kg animal, Dormicum, Hoffman-La Roche, Basel, Switzerland) and Fluanisone/Fentanyl (41/9 mg/kg animal respectively, Hypnorm, Janssen, Beerse, Belgium). All experimental procedures were performed in agreement with the Animal Ethics Committee in Lund, Sweden. The experiments were performed at ZT3 (9 am) and ZT15 (9 pm) in regard to glucose homeostasis after meal challenge and during hypoglycemia. Some data were also collected at ZT9 (3 pm) and ZT21 (3 am).

2.2. Mixed meal tolerance test (MTT)

The MTT was performed following 5 h of fasting. A 60/20/20E% Glucose/Protein/Lipid mixed meal solution was administered as a 500 μL gavage as previously described [16]. Blood samples were collected from the retrobulbar intraorbital capillary plexus before (0 min) and at 15, 30, 45 and 60 min in the experimental series for measurements of insulin or at 5, 10 and 20 min in the experimental series for measurements of glucagon following oral gavage. Plasma samples for glucose and hormone determination were stored at −20° awaiting analysis.

2.3. Hypoglycemic hyperinsulinemic clamp

The hypoglycemic clamp was performed following 5 h of fasting. Surgery and clamp experiments were performed as previously described [17] with the protocol modification of returning of red blood cells [18]. Briefly, the right jugular vein and the left carotid artery were catheterized using catheters filled with heparinized saline (100 U/mL). The mice remained anesthetized to reduce variation in the blood glucose concentrations due to stress. Following baseline sampling, synthetic human insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark) was infused as a continuous infusion (15 mU/kg animal/min) at a pace of 2 μL/min for 90 min. Blood glucose in 5 μL whole blood was determined every 10 min with an Accu-Chek Aviva blood glucose monitor (Hoffman-LaRoche). A variable amount of a 10% glucose (Sigma–Aldrich, MO, USA) solution was infused to maintain blood glucose levels at 2.5 mmol/L. Glucose requirement to maintain target glucose was represented by the glucose infusion rate (GIR) during the final 30 min steady state of the clamp.

2.4. Islet experiments

Pancreatic islets were isolated at ZT3 and ZT15 by collagenase digestion and handpicked under the microscope. Batches of freshly isolated islets were pre-incubated in HEPES balanced salt solution containing 125 mmol/L NaCl, 5.9 mmol/l KCL, 1.28 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 25 mmol/L HEPES (pH 7.4), 5.6 mmol/L glucose and 0.1% fatty acid free BSA (Boehringer Mannheim, Mannheim, Germany) at 37 °C during 60 min. Thereafter, islets in groups of three were incubated in 200 μl of the above described buffer but with 2.8 and 11.1 mM glucose without or with addition of arginine (10 mM) at 37 °C during 60 min. Aliquots of the buffer were collected and stored at −20 °C until analysis of insulin levels.

2.5. Analysis

Plasma glucose during the MTT was measured with the glucose oxidase method. Plasma and medium insulin was analysed with sandwich immunoassay technique (ELISA; Mercodia, Uppsala, Sweden) using double monoclonal antibodies according to manufacturer's protocol. Plasma glucagon was analyzed with ELISA (Mercodia), using double monoclonal antibodies, according to manufacturer's protocol.

2.6. Calculations and statistics

All data are presented as mean ± S.E.M. Basal insulin sensitivity during MTT was determined with the quantitative insulin sensitivity check index (QUICKI) which has been well validated in mice [19]. Clamp insulin sensitivity (SI_{Clamp}) and glucose clearance per unit of insulin (Cl_{Clamp}) was calculated as previously described [20]. Comparisons between groups were performed using a two-tailed Student's *t*-test (paired when applicable) or a 2-way ANOVA with a Holm–Sidak's multiple comparison test post hoc. Comparisons within groups between time points were performed using repeated measure ANOVA and difference from time point 0 min was calculated post hoc using Holm–Sidak's multiple comparison test. Incremental area under the curve (iAUC) was calculated using the trapezoidal rule.

3. Results

3.1. Glucagon response to meal ingestion in normal mice

Whereas baseline blood glucose did not differ between ZT3 and ZT15 (Fig. 1A), glucose excursion after MTT was lower at ZT3 compared to ZT15 at 10 min (Fig. 1A). In contrast, there was no significant difference in the glucagon response to MTT between ZT3 and ZT15 (Fig. 1B,C).

3.2. Glucagon response to hypoglycemia in normal mice

To study the glucagon response to hypoglycemia, hyperinsulinemic hypoglycemic clamp at 2.5 mmol/L was undertaken at ZT3 and ZT15 in normal mice; at this glucose level a robust glucagon response is provoked [18]. Basal blood glucose or blood glucose during the clamp did not differ between ZT3 and ZT15 (Fig. 2A) but the GIR needed to maintain target blood glucose of 2.5 mmol/L was significantly lower at ZT3 compared to ZT15 (Fig. 2B,C). Consequently, insulin sensitivity (SI_{Clamp}) was

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