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Published in:
Peptides

DOI:
10.1016/j.peptides.2016.01.008

2016

Document Version:
Peer reviewed version (aka post-print)

Link to publication

Citation for published version (APA):
Malmgren, S., & Ahrén, B. (2016). Evidence for time dependent variation of glucagon secretion in mice. *Peptides*, *76*, 102-107. https://doi.org/10.1016/j.peptides.2016.01.008

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Evidence for diurnal variability of glucagon secretion in mice

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Running title: Glucagon signaling and diurnal variation

Word count: 2612; Number of tables and figures: 4

Key words: Glucagon, glucose, oscillation, diurnal rhythm, hypoglycemia, 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 hypoglycemia 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 glucagon secretion displays a diurnal variability which is dependent both on intraislet and extraislet regulatory mechanisms in normal mice 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.

1. Introduction

Glucose metabolism displays circadian rhythm which is partially a result of dietary intake during the active phase of the 24h 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 hour 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 subjects 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 compared the glucagon response to hypoglycemia and meal test, and glucagon secretion from isolated islets between *zetigeber* 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 12h 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 respectably, Hypnorm, Janssen, Beerse, Belgium). All experimental procedures were performed in agreement with the Animal Ethics Committee in Lund, Sweden.The experiments were performed at ZT 3 (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 minutes. Blood glucose in ~5 µL whole blood was determined every 10 minutes 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 (Figs. 1B and 1C).

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 (Figs. 2B and 2C). Consequently, insulin sensitivity (SI_{Clamp}) was higher at ZT15 than at ZT3 (4.8±0.9 vs 1.5±0.2 L/kg x min, p=0.003, Fig. 2E) and so was glucose clearance per unit of insulin (2.1±0.5 vs 0.6±0.1 L^2/kg x min x mmol, p=0.006; Fig. 2F). The glucagon response to hypoglycemia was significantly higher at ZT15 than at ZT3, both when measured in absolute concentrations (7.5±1.2 vs 3.3±1.5 pmol/L, p=0.019; Fig. 2G) and when estimated as fold change over basal (4.8±1.2 vs 1.9±0.54, p=0.035; Fig. 2H).

3.3 *Glucagon secretion from isolated islets*

Glucagon secretion from isolated islets from normal mice at 2.8 or 11.1 mmol/L was not different at ZT3 versus ZT15. However, glucagon secretion in response to 10 mmol/L arginine was higher at ZT3 than at ZT15 both at 2.8 mmol/L and 11.1 mmol/L glucose (both $p<0.001$).

3.4 GCGR knockout alters the circadian rhythm of metabolism

GCGR-/- mice had lower circulating glucose than their wt littermates at ZT3 (4.2±0.2 versus 7.4±0.3 mmol/L, $p<0.001$) and ZT9 (5.5±0.2 versus 7.6 ±0.2 mmol/L, $p=0.0002$) but not at ZT15 (5.2±0.2 versus 5.4±0.1 mmol/L; Figs. 3A-C). GCGR-/- mice had also a lower insulin response to meal than wt mice at ZT3 and ZT9 but not at ZT15 (Figs. 3D-G). Insulin sensitivity, measured as QUICKI after meal ingestion, was lower in GCGR-/- than in wt mice at ZT3 and ZT9, but not at ZT15 (Fig. 3H).

4. Discussion

As most species, both mice and humans exhibit oscillatory patterns in behavior and physiological functions over the course of the day (1). Central and peripheral gene clocks regulate this and they are in turn regulated by the effect of light on the retina of the eye (4,5), by food intake (21,22) as well as by specific metabolic hormones (23). In this study, we have explored the potential role of glucagon in this respect by examining the glucagon secretion during hypoglycemia and after meal ingestion as well as in vitro at ZT3 versus ZT15 in normal C57BL/6J mice and basal and postprandial glucose levels were also examined at these time points in GCGR-/- mice and their wildetype (wt) littermates.

A main general novel finding of this study is that there indeed is a diurnal variability in glucagon secretion in normal mice. The detail of this variability is, however, dependent on the experimental condition. Thus, whereas the glucagon counterregulation to hypoglycemia is lower at ZT3 than at ZT15, arginine-stimulated glucagon secretion from isolated islets shows the opposite pattern, being higher at ZT3 than at ZT15, and glucose-dependent glucagon secretion from islets and the glucagon secretion to meal ingestion is the same at ZT3 and ZT15. Therefore, the diurnal variability in glucagon secretion is complex and regulated both by islet and extraislet mechanisms since many factors regulate glucagon secretion besides the capacity in the islet alpha cells.

To study glucagon secretion during hypoglycemia we used our recently developed hypoglycemic clamp in mice, where we demonstrated a clear glucagon response when glucose levels were reduced (18). The glucagon response under this condition is complexly regulated by secretory capability from the alpha cells when glucose levels are reduced in combination with stimulation by other counter-regulatory hormones, such as epinephrine released from the adrenals, and the autonomic nerves (24). Since we did not observe any diurnal variability in the effect of low glucose on glucagon secretion from isolated islets between ZT3 and ZT15, our conclusion is that the variability during hypoglycemia is not dependent on different glucose sensitivity in alpha cells. Instead, the difference between the light and dark phase in glucagon response to hypoglycemia may rather be caused by a diurnal variation in the other counterregulatory hormones. The lower glucagon at ZT3 compared to ZT15 during hypoglycemia coincided with a lower insulin sensitivity as judged by a lower glucose infusion rate to maintain the target hypoglycemic glucose level during the clamp at ZT3. This may suggest a cross-talk between insulin sensitivity and glucagon secretion such that when insulin sensitivity is lower, the requirement for glucagon to restore hypoglycemia is more limited.

To examine the potential of glucagon variability during another physiological condition we used a mixed meal test, by applying a recently developed model when a mixture of glucose, fat and protein resembling a mixed meal was administered to mice (16). In this model, glucagon secretion is stimulated, which is mainly achieved by a combination of fatty acids and amino acids derived from the meal constituents. We found that there was no difference in the glucagon response to meal ingestion when we compared ZT3 and ZT15, suggesting that in contrast to the glucagon counterregulation to hypoglycemia, there is no evidence of a diurnal variability in the glucagon response to meal ingestion. It was therefore a surprise when we documented a clear diurnal variability in glucagon secretion from islets in response to arginine, both at low and high glucose, with a higher glucagon secretion at ZT3 than at ZT15. This shows an interesting diurnal variability in the capacity to secrete glucagon, which is not reflected in a similar difference in vivo with a more modest stimulation. The mechanism and potential contribution of this diurnal variability needs now to be examined in more detail.

To examine whether the diurnal variability in glucagon secretion is important for glucose homeostasis, we assessed the hormonal response to a meal ingestion in GCGR-/- mice and wt controls. As reported previously, a characteristic phenotype in GCGR-/- mice is lower baseline glucose, impaired insulin secretion after arginine stimulation and enhanced insulin sensitivity (15,25). We confirm here that these mice, compared to their wt littermates, have reduced baseline glucose, reduced insulin response to meal ingestion and increased insulin sensitivity. However, the main finding in this respect is that these phenotype characteristics were evident only at ZT3 and not seen at ZT15. This further suggests a potential contribution of glucagon to diurnal variability of glucose homeostasis. However, the mechanisms explaining these discrepancies between ZT3 and ZT15 in GCGR-/- mice remain to be established. Due to hyper-production of pro-glucagon, the GCGR $^{-/-}$ animals have increased levels of both glucagon and glucagon-like peptide-1 (GLP-1) (25), which might contribute to the phenotype in these animals. However, although recent studies have suggested that GLP-1 has a circadian rhythm (26), it remains yet to be shown whether it affects peripheral gene clocks. It would be interesting to test this, as a recent paper did (27), in a double knockout model. Nevertheless, a consequence of our findings is that the well known phenotype of these mice with reduction of glucose levels and impaired insulin secretion (15,25) depends on the time of the day when it is measured. In a broader perspective, this raises questions on results derived from other hormone-altering mouse models where a diurnal variation in glucose homeostasis might exaggerate or occlude the phenotype and it highlights the importance of time dependent effects in metabolic phenotyping. Furthermore, the true circadian rhythm in glucose homeostasis with particular attention to glucose needs now to be tested over the entire 24 hr period in at least two cycles.

In conclusion, glucagon secretion displays a diurnal variability which is dependent both on intraislet and extraislet regulatory mechanisms in normal mice and, furthermore, the phenotype characteristics of a lower glucose, reduced insulin response to meal and lower insulin sensitivity in GCGR-/- mice are evident only during the light phase. Our results therefore suggest that there is a link between glucagon signaling and the diurnal variation of glucose homeostasis and that the phenotype of the GCGR-/- mice is dependent on the time of the day when it is examined.

ARTICLE INFORMATION

Author contributions. S.M designed study, performed experiments, analyzed data, drafted and wrote manuscript and the final version of manuscript. B.A. designed study, analyzed data, wrote and revised manuscript and approved final version. B.A is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgments. The authors would like to thank Kristina Andersson of Lund University, Sweden, for experimental expertise. Furthermore, we extend our gratitude to Novo Nordisk for providing the GCGR-/- mice.

Funding. This study was supported by grants from Kungl. Fysiografiska Sällskapet i Lund, the Swedish Medical Research Council (grant 6834), Region Skåne and Lund University.

Duality of Interest. The authors have nothing to disclose in relation to this study.

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FIGURE LEGENDS

Figure 1—Plasma glucose (A) and glucagon levels (B), and fold change increase in glucagon compared to basal (0 min; C) during a MTT in female C57BL/6J mice at ZT3 (open circle) or at ZT15 (square). Mean±SEM are shown, n=7 for each group, *p<0.05 paired comparison between groups.

Figure 2—Blood glucose levels (A), cumulated glucose infusion (B) and steady state glucose infusion rate (GIR;C) during a hyperinsulinemic hypoglycemic clamp in female C57BL/6J mice at ZT3 (open circle/white bars) or at ZT15 (square/black bars). Steady state glucose is obtained during the last 30 minutes of the experiment. Insulin levels (D), insulin sensitivity index (SI; E), glucose clearance per unit of insulin (Cl; F), glucagon levels (G) and fold change increase in glucagon compared to basal (H) during hyperinsulinaemic hypoglycaemic at ZT3 (open circle/white bars) or at ZT15 (square/black bars). Mean±SEM are shown, n=8 for each group,

*p<0.05, **p<0.01, ***p<0.001, *p<0.05 comparison between groups, $^{\#}$p<0.05 compared to basal (0 min) for each group.

Figure 3—Plasma glucose levels (A-C), iAUC of insulin levels (D), plasma insulin levels (E-G) and insulin sensitivity measured through QUICKI (H) during a MTT in female C57BL/6J mice at ZT3 (A and E), ZT9 (B and F) and ZT15 (C and G) in GCGR-/- (square/striped bar) and wt mice (circle/white bar). Mean±SEM are shown, n=18-20 for each group, *p<0.05, **p<0.01, ***p<0.001, *p<0.05 comparison between groups, $^{\#}$p<0.05 compared to ZT15 minutes for each group.

Table 1 Glucagon secretion from isolated islets from C57BL/6J mice after incubation for 1 hr in glucose at 2.8 mmol/L or 11.1 mmol/L without or with addition of aarginine at 10 mmol/L at ZT3 or ZT15. A total of 24 incubations with 3 islets in each from 3 mice were performed. Means ± S.E.M. are shown.

	Glucagon ZT3 (pg/islet/hr)	Glucagon ZT15 (pg/islet/hr)
2.8 mmol/L glucose	3.9±0.7	3.6±0.7
2.8 mmol/L glucose +10 mmol/L arginine	9.2±1.0	6.3±0.9
11.1 mmol/L glucose	3.0±0.4	3.1±1.0
11.1 mmol/L glucose + 10 mmol/L arginine	5.3±0.7	2.4±0.3

FIGURES

Figure 1

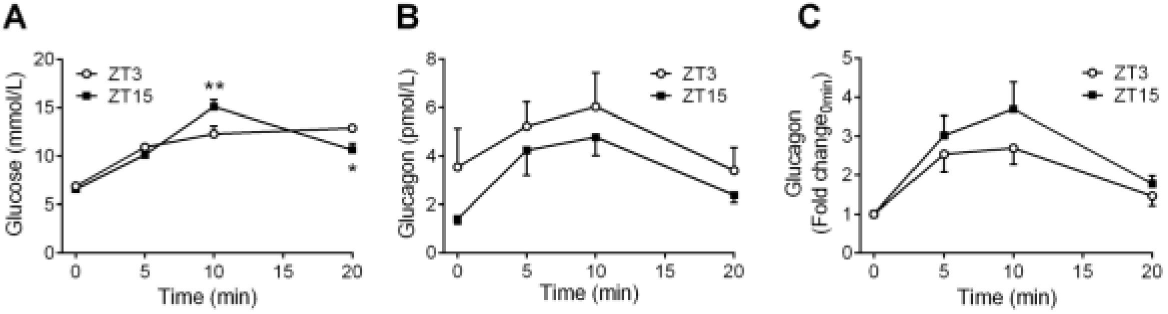


Figure 2

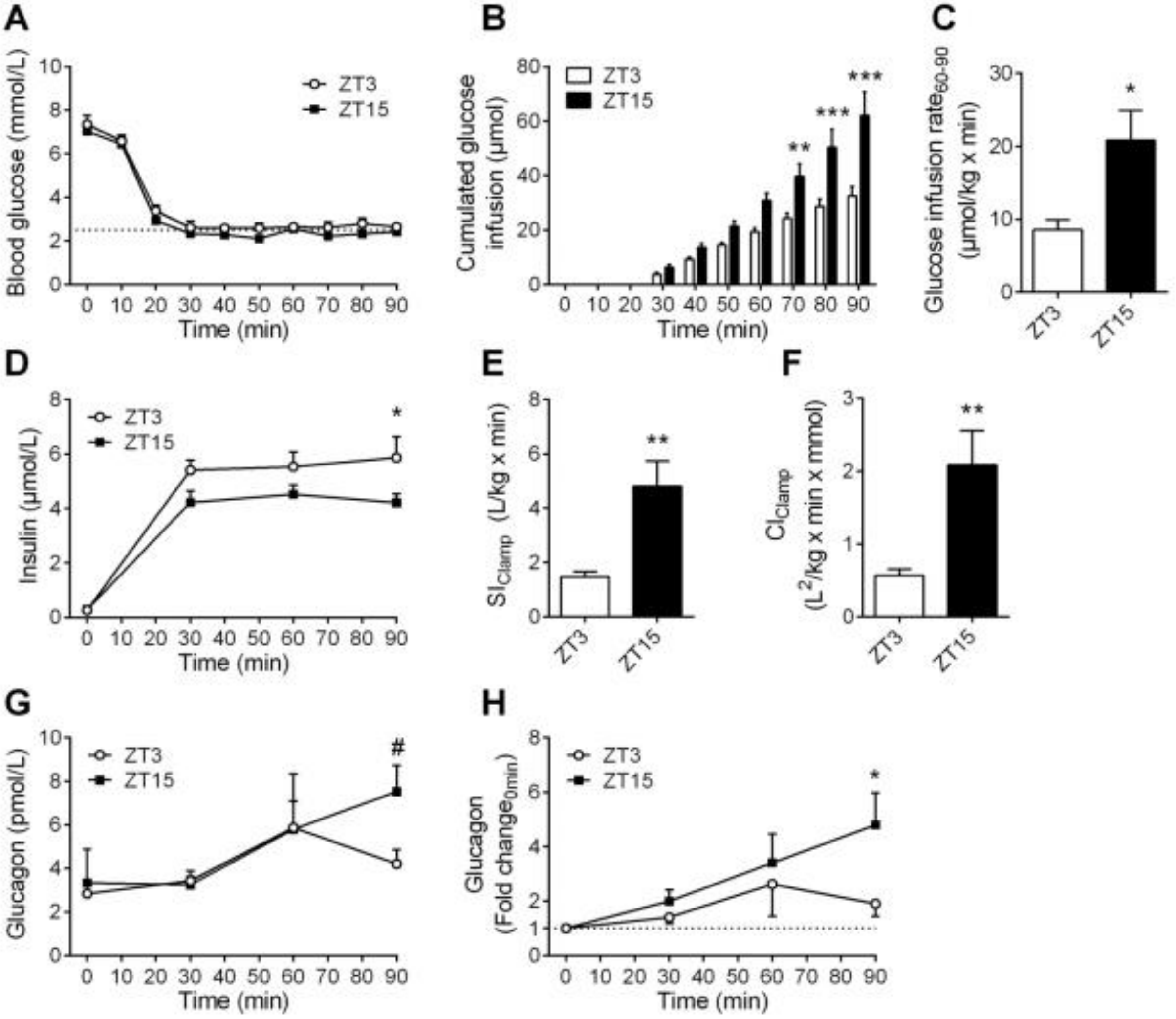
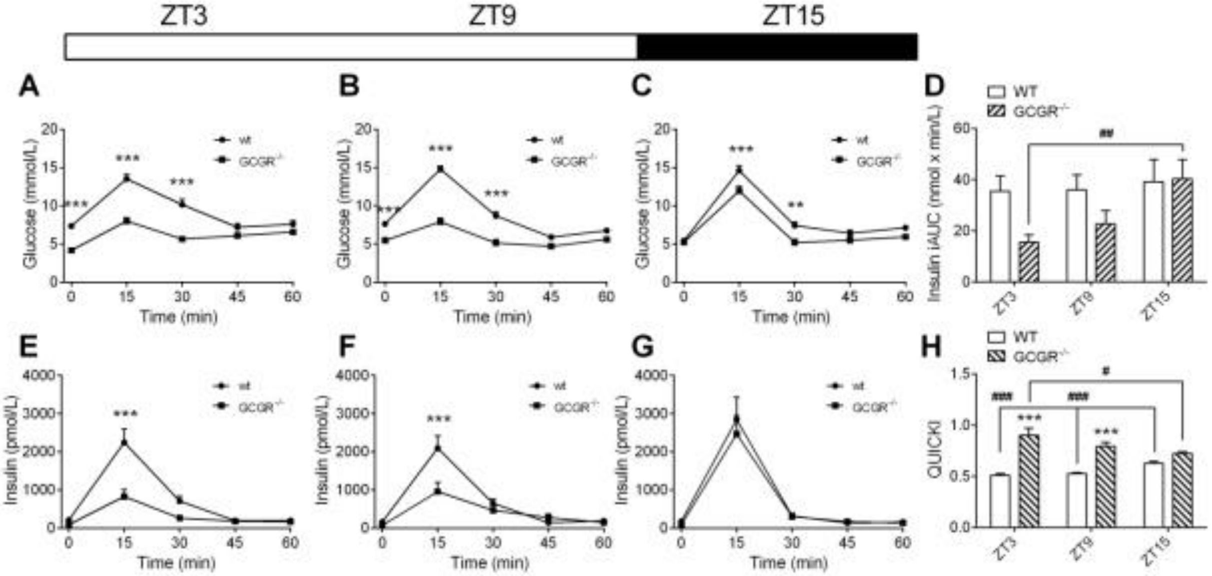


Figure 3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