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Evidence for diurnal variability of glucagon secretion in mice

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24

25

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.

47 **1. Introduction**

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

61 The importance of the clock genes for glucose homeostasis is evident by findings that genetic
62 deletion of the clock transcription factor in the hypothalamus in mutant mice alters the diurnal
63 feeding pattern and results in overeating, obesity and a sign of metabolic syndrome
64 characterized with hyperglycemia and insulin deficiency (8). Furthermore, lesion in the
65 suprachiasmatic nuclei disrupts the circadian rhythm of glucose and insulin in mice (9).
66 Moreover, disruption in the transcription of *Clock* and *Bmal1* alters the expression of genes
67 essential to beta cell function and leads to insulin deficiency and diabetes (10). The importance
68 of the clock system for glucose homeostasis and islet function is also emphasized by findings

69 that an autonomic rhythm exists within pancreatic beta cells (11,12) and that conditional
70 disruption of the clock in the pancreas results in impaired beta cell function and diabetes (13).

71 Recently, it was demonstrated that the pancreatic glucagon producing alpha cells is regulated
72 by the clock gene *Rev-erb alpha* such that silencing of this gene inhibits glucagon secretion
73 whereas a *Rev-erb alpha* agonist stimulates glucagon secretion (14). This would suggest that not
74 only insulin but also glucagon is the subjects of diurnal variation through clock regulation. This
75 would be of interest since glucagon stimulates hepatic glucose production which is a key
76 mechanism for preventing hypoglycemia during the inactive phase (1). Interestingly, it has also
77 been reported but not widely discussed, that the phenotype characteristic of a reduction in
78 circulating glucose in glucagon receptor knockout (GCGR-/-) mice is observed only in the
79 morning hours and vanishes later during the day (15), which may further indicate that glucagon
80 is involved in the diurnal variation of glucose homeostasis.

81 However, besides these studies there is little evidence linking glucagon signaling or glucagon
82 secretion to diurnal variation of glucose homeostasis. To gain further insight in the potential
83 involvement of glucagon in this respect, we compared the glucagon response to hypoglycemia
84 and meal test, and glucagon secretion from isolated islets between *zetigeber* time (ZT) 3 and
85 ZT15 in normal mice and compared glucose levels and insulin response to meal in GCGR-/- mice
86 and their wildtype littermates.

87

88 ## 2. Materials and methods

89 *2.1 Animals and anesthesia*

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

100 *2.2 Mixed meal tolerance test (MTT)*

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

107 *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

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

135 *2.6 Calculations and statistics*

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

145

146 **3. Results**

147 *3.1 Glucagon response to meal ingestion in normal mice*

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

152 *3.2 Glucagon response to hypoglycemia in normal mice*

153 To study the glucagon response to hypoglycemia, hyperinsulinemic hypoglycemic clamp at 2.5
154 mmol/L was undertaken at ZT3 and ZT15 in normal mice; at this glucose level a robust glucagon
155 response is provoked (18). Basal blood glucose or blood glucose during the clamp did not differ
156 between ZT3 and ZT15 (Fig. 2A) but the GIR needed to maintain target blood glucose of 2.5
157 mmol/L was significantly lower at ZT3 compared to ZT15 (Figs. 2B and 2C). Consequently,
158 insulin sensitivity (SI_{Clamp}) was higher at ZT15 than at ZT3 (4.8±0.9 vs 1.5±0.2 L/kg x min,
159 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
160 x mmol, p=0.006; Fig. 2F). The glucagon response to hypoglycemia was significantly higher at
161 ZT15 than at ZT3, both when measured in absolute concentrations (7.5±1.2 vs 3.3±1.5 pmol/L,
162 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;
163 Fig. 2H).

164 3.3 *Glucagon secretion from isolated islets*

165 Glucagon secretion from isolated islets from normal mice at 2.8 or 11.1 mmol/L was not
166 different at ZT3 versus ZT15. However, glucagon secretion in response to 10 mmol/L

167 arginine was higher at ZT3 than at ZT15 both at 2.8 mmol/L and 11.1 mmol/L glucose (both
168 $p<0.001$).

169 *3.4 GCGR knockout alters the circadian rhythm of metabolism*

170 GCGR-/- mice had lower circulating glucose than their wt littermates at ZT3 (4.2±0.2 versus
171 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
172 (5.2±0.2 versus 5.4±0.1 mmol/L; Figs. 3A-C). GCGR-/- mice had also a lower insulin response to
173 meal than wt mice at ZT3 and ZT9 but not at ZT15 (Figs. 3D-G). Insulin sensitivity, measured as
174 QUICKI after meal ingestion, was lower in GCGR-/- than in wt mice at ZT3 and ZT9, but not at
175 ZT15 (Fig. 3H).

176

177 **4. Discussion**

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

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

195 To study glucagon secretion during hypoglycemia we used our recently developed hypoglycemic
196 clamp in mice, where we demonstrated a clear glucagon response when glucose levels were
197 reduced (18). The glucagon response under this condition is complexly regulated by secretory

198 capability from the alpha cells when glucose levels are reduced in combination with stimulation
199 by other counter-regulatory hormones, such as epinephrine released from the adrenals, and the
200 autonomic nerves (24). Since we did not observe any diurnal variability in the effect of low
201 glucose on glucagon secretion from isolated islets between ZT3 and ZT15, our conclusion is that
202 the variability during hypoglycemia is not dependent on different glucose sensitivity in alpha
203 cells. Instead, the difference between the light and dark phase in glucagon response to
204 hypoglycemia may rather be caused by a diurnal variation in the other counterregulatory
205 hormones. The lower glucagon at ZT3 compared to ZT15 during hypoglycemia coincided with a
206 lower insulin sensitivity as judged by a lower glucose infusion rate to maintain the target
207 hypoglycemic glucose level during the clamp at ZT3. This may suggest a cross-talk between
208 insulin sensitivity and glucagon secretion such that when insulin sensitivity is lower, the
209 requirement for glucagon to restore hypoglycemia is more limited.

210

211 To examine the potential of glucagon variability during another physiological condition we used
212 a mixed meal test, by applying a recently developed model when a mixture of glucose, fat and
213 protein resembling a mixed meal was administered to mice (16). In this model, glucagon
214 secretion is stimulated, which is mainly achieved by a combination of fatty acids and amino
215 acids derived from the meal constituents. We found that there was no difference in the
216 glucagon response to meal ingestion when we compared ZT3 and ZT15, suggesting that in
217 contrast to the glucagon counterregulation to hypoglycemia, there is no evidence of a diurnal
218 variability in the glucagon response to meal ingestion. It was therefore a surprise when we
219 documented a clear diurnal variability in glucagon secretion from islets in response to arginine,

220 both at low and high glucose, with a higher glucagon secretion at ZT3 than at ZT15. This shows
221 an interesting diurnal variability in the capacity to secrete glucagon, which is not reflected in a
222 similar difference in vivo with a more modest stimulation. The mechanism and potential
223 contribution of this diurnal variability needs now to be examined in more detail.

224 To examine whether the diurnal variability in glucagon secretion is important for glucose
225 homeostasis, we assessed the hormonal response to a meal ingestion in GCGR-/- mice and wt
226 controls. As reported previously, a characteristic phenotype in GCGR-/- mice is lower baseline
227 glucose, impaired insulin secretion after arginine stimulation and enhanced insulin sensitivity
228 (15,25). We confirm here that these mice, compared to their wt littermates, have reduced
229 baseline glucose, reduced insulin response to meal ingestion and increased insulin sensitivity.
230 However, the main finding in this respect is that these phenotype characteristics were evident
231 only at ZT3 and not seen at ZT15. This further suggests a potential contribution of glucagon to
232 diurnal variability of glucose homeostasis. However, the mechanisms explaining these
233 discrepancies between ZT3 and ZT15 in GCGR-/- mice remain to be established. Due to hyper-
234 production of pro-glucagon, the GCGR $^{-/-}$ animals have increased levels of both glucagon and
235 glucagon-like peptide-1 (GLP-1) (25), which might contribute to the phenotype in these animals.
236 However, although recent studies have suggested that GLP-1 has a circadian rhythm (26), it
237 remains yet to be shown whether it affects peripheral gene clocks. It would be interesting to
238 test this, as a recent paper did (27), in a double knockout model. Nevertheless, a consequence
239 of our findings is that the well known phenotype of these mice with reduction of glucose levels
240 and impaired insulin secretion (15,25) depends on the time of the day when it is measured. In a
241 broader perspective, this raises questions on results derived from other hormone-altering

242 mouse models where a diurnal variation in glucose homeostasis might exaggerate or occlude
243 the phenotype and it highlights the importance of time dependent effects in metabolic
244 phenotyping. Furthermore, the true circadian rhythm in glucose homeostasis with particular
245 attention to glucose needs now to be tested over the entire 24 hr period in at least two cycles.

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

252

253

254 ## ARTICLE INFORMATION

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

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

266

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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,

351 *p<0.05, **p<0.01, ***p<0.001, *p<0.05 comparison between groups, #p<0.05 compared to

352 basal (0 min) for each group.

353 **Figure 3**—Plasma glucose levels (A-C), iAUC of insulin levels (D), plasma insulin levels (E-G) and

354 insulin sensitivity measured through QUICKI (H) during a MTT in female C57BL/6J mice at ZT3 (A

355 and E), ZT9 (B and F) and ZT15 (C and G) in GCGR-/- (square/striped bar) and wt mice

356 (circle/white bar). Mean±SEM are shown, n=18-20 for each group, *p<0.05, **p<0.01,

357 ***p<0.001, *p<0.05 comparison between groups, #p<0.05 compared to ZT15 minutes for each

358 group.

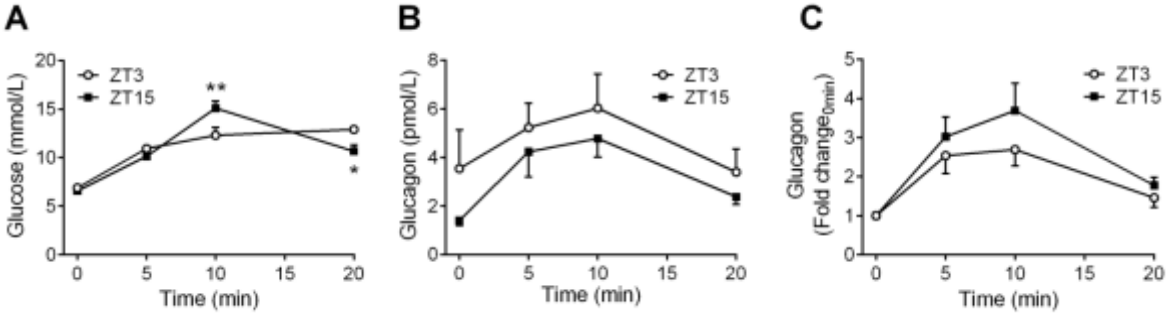
359

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

368 **FIGURES**

369 **Figure 1**



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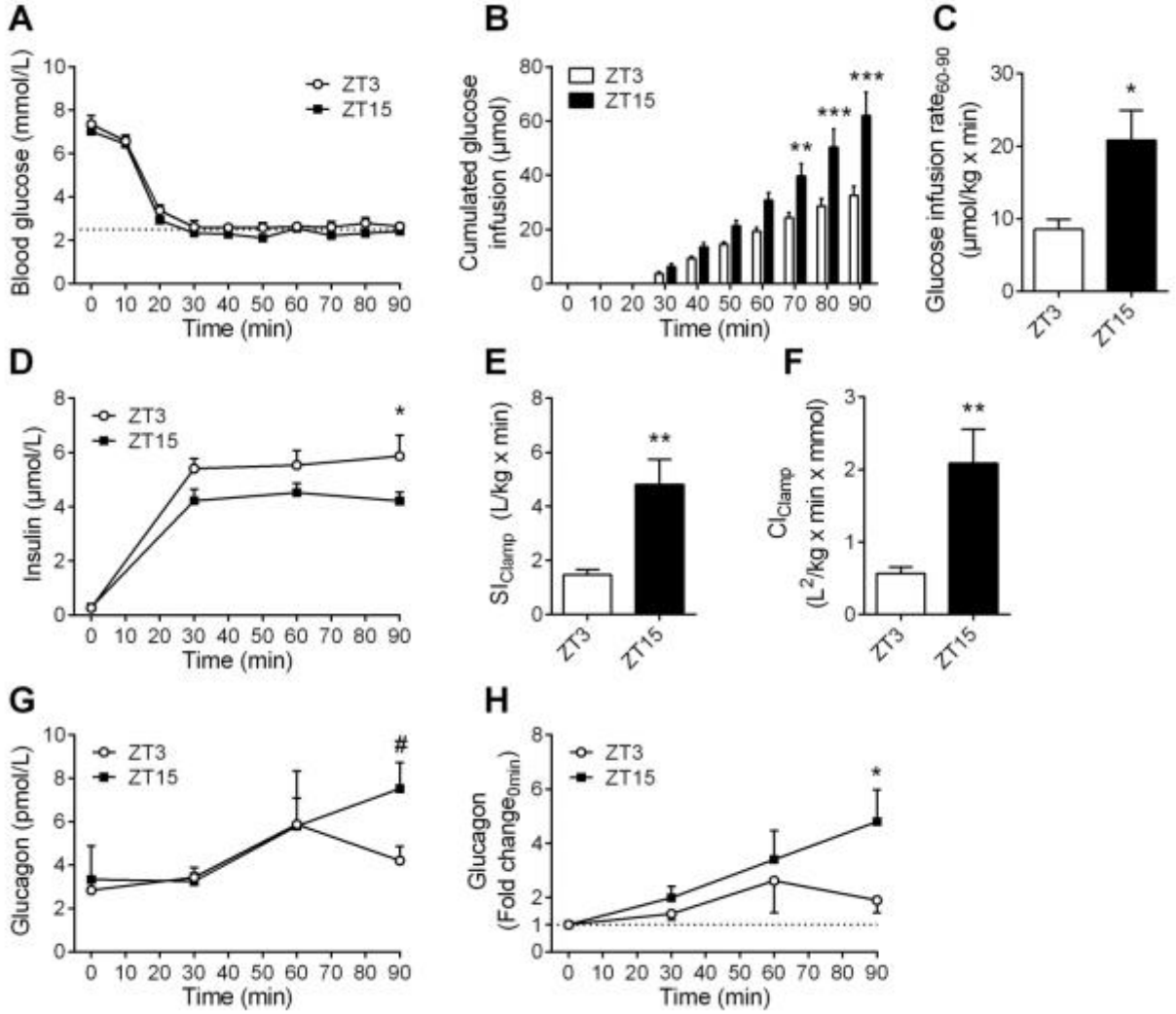
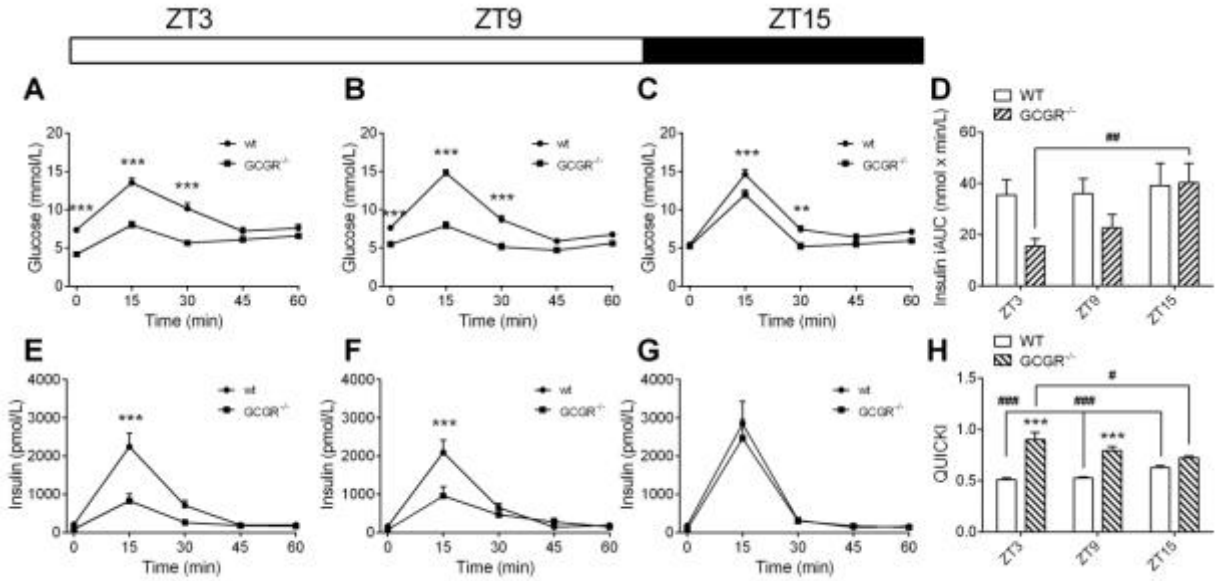
371 **Figure 2**



372

373 **Figure 3**

374



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