Hepatic Metabolic Memory Triggered by AM Exposure to Glucagon Alters Afternoon Glucose Metabolism

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Abstract

The second meal effect describes an improved glycemic response observed after consuming a second identical meal. We previously showed that morning (AM) exposure to hyperinsulinemia primes the liver for enhanced hepatic glucose uptake and glycogen storage in the afternoon (PM), with no significant effect on PM non-hepatic glucose uptake. Given that meals often trigger both insulin and glucagon secretion, we aimed to determine if AM hyperglucagonemia alters the priming effect of AM hyperinsulinemia on PM hepatic glucose metabolism. To test this, dogs were exposed to a 4h AM hyperinsulinemic-euglycemic clamp, with insulin delivered in a pattern mimicking the insulin profile observed earlier during a 4h AM duodenal glucose infusion. This period of hyperinsulinemia was paired with either basal (Prime, n=8) or elevated (Prime + ↑GGN, n=8) glucagon, maintaining a consistent insulin-to-glucagon molar ratio throughout the AM clamp. After a 1.5h rest period, the dogs underwent a 2.5h PM hyperinsulinemic-hyperglycemic clamp, during which glucose, insulin, and glucagon levels, along with the artery-to-portal vein glucose gradient, were carefully controlled to replicate postprandial conditions. During the PM clamp, the mean net hepatic glucose uptake (NHGU) in the Prime + ↑GGN group was only 59% of that in the Prime group (3.6±0.4 vs. 6.1±0.6 mg/kg/min, $P<0.0027$, respectively). Additionally, PM direct glycogen synthesis was two-fold greater in the Prime group compared to the Prime + ↑GGN group (3.2±0.7 vs. 1.5±0.2 mg/kg/min, $P<0.0014$, respectively). The observed difference in PM NHGU between the groups was not due to enhanced PM hepatic glucose uptake (HGU), which was similar in both groups (5.7±0.5 mg/kg/min in the Prime group vs. 5.2±0.3 mg/kg/min in the Prime + ↑GGN group), but rather a prolonged effect of AM hyperglucagonemia on PM hepatic glucose production (HGP) (−0.3±0.3 mg/kg/min in the Prime group vs. 1.7±0.4 mg/kg/min in the Prime + ↑GGN group, $P<0.0072$). This increase in PM HGP in the Prime + ↑GGN group was not driven by differences in PM gluconeogenic flux but by futile glucose cycling between glucose and glucose-6-phosphate, as well as hepatic glycogen storage and breakdown. In summary, these findings

suggest that morning exposure to elevated glucagon shifts the insulin-driven priming effect on afternoon hepatic glucose metabolism by promoting sustained glucose cycling at the expense of glycogen synthesis and glycolysis, leading to persistent HGP despite identical PM insulin, glucose, and glucagon levels.

Introduction

The metabolic response to the first meal of the day has profound effects on glucose regulation during subsequent meals, often resulting in improved postprandial glucose handling during the second meal (1-3). This response, known as the second meal phenomenon**,** is primarily driven by the priming of afternoon HGU and glycogen storage**,** which are largely influenced by morning hepatic exposure to insulin (4). Notably, this priming effect appears to be liver-specific, as it does not significantly impact non-hepatic glucose uptake. While insulin is a crucial driver of this response, other hormonal and metabolic factors may also contribute, yet their precise role remains unclear.

One key factor that may influence the second meal phenomenon is glucagon**,** which opposes insulin by promoting HGP through stimulation of glycogenolysis and gluconeogenesis (5; 6)**.** Previous studies have demonstrated that higher glucagon levels reduce hepatic glucose uptake and glycogen synthesis, even under conditions of high insulin (7; 8). Given that both insulin and glucagon are secreted in response to meals**,** their interaction is likely critical in shaping postprandial glucose metabolism throughout the day (9; 10). In individuals with prediabetes and type 2 diabetes (T2DM), the glucagon response to a mixed meal is often exaggerated compared to normoglycemic individuals and is particularly exacerbated after meals high in protein (10; 11). Elevated glucagon levels in these individuals have been linked to impaired glucose homeostasis and an increased risk of T2DM progression (12-14)**.** While

glucagon's role in acute postprandial glucose regulation is well characterized, its prolonged effects on later metabolic responses remain poorly understood.

Therefore, this study aimed to determine whether morning hyperglucagonemia had the potential to impact the insulin-driven priming effect on afternoon hepatic glucose metabolism. Given that the insulin-to-glucagon ratio governs metabolic balance, an elevation in glucagon concurrent with hyperinsulinemia very well may alter the liver's metabolic programming across meals. We hypothesized that morning hyperglucagonemia attenuates the insulin-driven priming effect on afternoon hepatic glucose metabolism, potentially limiting the benefits of insulin priming seen with a high-carbohydrate meal. By directly assessing these effects, we aim to clarify the interaction between insulin and glucagon across meals and determine how hormonal fluctuations shape hepatic glucose handling over time. These insights are critical for understanding the second meal effect and informing strategies to improve glycemic control in metabolic diseases such as T2DM.

Research Design and Methods

Animal care and surgical procedures

The study was conducted on 16 adult mongrel dogs (9 males, 7 females) with an average weight of 25.2 ± 0.8 kg. The animals were sourced from a USDA-licensed vendor and housed in compliance with the American Association for the Accreditation of Laboratory Animal Care guidelines. All experimental procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee. Two weeks before each experiment, a laparotomy was performed under general anesthesia to surgically place blood flow probes around the hepatic artery and hepatic portal vein. Catheters for blood sampling were inserted into the hepatic vein, hepatic portal vein, and femoral artery, while infusion catheters were positioned in the splenic and jejunal veins (which supply the hepatic portal vein) and the inferior vena cava. These

catheters were secured subcutaneously until the study day. The dogs were maintained on a controlled diet consisting of chow and meat, providing 46% carbohydrate, 34% protein, 14.5% fat, and 5.5% fiber. They underwent an 18-hour fasting period before the experiment. Health status was assessed based on meal intake (>75% of the most recent meal), leukocyte count (<18,000/mm³), and hematocrit level (>34%). Blood sampling was restricted to no more than 20% of total blood volume.

Experimental Design

The 8-hour experimental protocol included two glucose clamping periods designed to mimic postprandial-like conditions at times corresponding to standard breakfast and lunch meals, denoted as the AM clamp (0-240 min) and the PM clamp (330-480 min), respectively **(Fig. 1)**. Blood samples were collected every 15–30 minutes from the femoral artery, hepatic portal vein, and hepatic vein to measure hormone and substrate levels. Arterial plasma glucose was monitored every 5 minutes, and exogenous glucose infusion into a peripheral vein was adjusted as needed to ensure precise glucose control.

Morning (AM) Clamp Period (0-240 min)

Previous studies established that the improvement in PM hepatic glucose uptake was not driven by a rise in glucose during the morning period but rather by an increase in insulin, which primes the liver to handle the PM glucose load more efficiently. Therefore, a hyperinsulinemic-euglycemic clamp was performed in the AM period. At the start of the clamp, somatostatin (Bachem, Torrance, CA) was infused into the inferior vena cava (0.8 µg/kg/min) to suppress endogenous pancreatic insulin and glucagon secretion. To replicate the physiologic insulin secretion pattern previously observed following a morning duodenal glucose infusion, insulin (Novolin R; Novo Nordisk, Basværd, Denmark) was infused intraportally at 2.1 mU/kg/min from 0-30 min, 2.4 mU/kg/min from 30-60 min, and 1.5 mU/kg/min from 60-240 min

(15). Two experimental groups were studied. One group (Prime) received a basal intraportal glucagon infusion (GlucaGen, Boehringer Ingelheim, Ridgefield, CT) at 0.57 ng/kg/min for 0-240 min, while the other group (Prime + ↑GGN) received an elevated, physiologic infusion of glucagon designed to maintain a consistent insulin-to-glucagon molar ratio, with infusion rates of 2.28 ng/kg/min from 0-30 min, 2.60 ng/kg/min from 30-60 min, and 1.63 ng/kg/min from 60–240 min. Glucose was clamped at euglycemia (~100 mg/dL plasma glucose). To allow for HGU measurements in the afternoon period, a peripheral infusion of [3-^{3}H] glucose (Revvitty, Waltham, MA) was initiated at 180 min in both groups, with a priming dose of 38 µCi, followed by a continuous infusion at 0.38 µCi/min. At the conclusion of the AM clamp (240 min), all infusions, except for the tracer, were discontinued.

Non-clamp Period (240 – 330 min)

To evaluate glucose kinetics before initiating the PM clamp, blood samples were collected from the femoral artery, portal vein, and hepatic vein catheters at 300, 315, and 330 min (Fig. 1). To examine the liver's molecular profile at the start of the PM phase, a subset of dogs (n=4/group) were anesthetized at 330 min, after which hepatic tissue was rapidly harvested, immediately flash-frozen in liquid nitrogen to preserve cellular conditions, and stored at -80°C. The remaining dogs (n=8/group) continued with the PM clamp protocol.

Afternoon (PM) Clamp Period (330 – 480 min

Both the Prime and Prime + ↑GGN groups underwent an identical 2.5-h hyperinsulinemic-hyperglycemic (HIHG) clamp during the afternoon phase **(Fig. 1).** Somatostatin was infused into a peripheral vein as described above, while basal glucagon and fourfold basal insulin (1.2 mU/kg/min) were administered intraportally. To activate the portal glucose feeding signal, glucose was infused directly into the hepatic portal vein (4 mg/kg/min). In addition, both groups received a primed, continuous glucose infusion into a peripheral vein to

quickly bring glucose levels up to hyperglycemia (~200 mg/dL plasma glucose). These glucose levels were maintained for the entirety of the PM clamp. These conditions were designed to replicate the steady-state metabolic environment following the ingestion of a carbohydrate-rich meal (16). After the final blood sample was obtained at 480 min, the dogs were anesthetized, and hepatic tissue was excised and preserved as previously described.

Analyses

Biochemical and Molecular Methods

Whole blood samples were utilized to assess hormone and substrate flux across the liver through arteriovenous balance techniques (17). During the experimental period, plasma glucose levels were measured in quadruplicate using an Analox GM9 glucose analyzer. Insulin (#PI-12K, MilliporeSigma, Burlington, MA), glucagon (#GL-32K, MilliporeSigma), and cortisol (VUMC Analytical Services in-house primary antibody with I^{125} cortisol from MP Biomedicals, Santa Ana, CA) were quantified via radioimmunoassay (17). Key metabolic intermediates, including lactate, glycerol, alanine, and non-esterified fatty acids (NEFA), were measured using enzymatic spectrophotometric assays (17). Plasma samples were deproteinized using the Somogyi method and quantified via liquid scintillation counting to determine the specific activity of [3-^{3}H]-glucose in each triple catheter sample (18; 19). Terminal hepatic glycogen was quantitatively assessed using the Keppler and Decker amyloglucosidase method (20).

Molecular analyses were performed on hepatic tissue collected at the end of the PM clamp, including qPCR analysis, western blotting, and enzymatic activity assays using radioisotope and colorimetric detection methods (17). Gene expression levels were normalized to GAPDH, while protein levels were standardized against either total protein or cyclophilin B, as appropriate. Assays were optimized for canine-specific targets using validated antibodies and primers (21). Additionally, baseline liver samples from five overnight-fasted dogs studied earlier were included as a reference.

Calculations

Net hepatic glucose balance (NHGB) was determined using arterio-venous difference calculations, expressed as NHGB = $[HGL_{out}] - [HGL_{in}]$. In this equation, HGL_{out} represents the glucose output from the liver and was computed as $[HGL_{out}] = [BF_h \times G_h]$, while HGL_{in} denotes the glucose entering the liver, calculated as $[HGL_{in}] = [(BF_a \times G_a) + (BF_p \times G_p)]$. Here, G represents blood glucose concentration, BF indicates measured blood flow, and A, P, and H correspond to the hepatic artery, hepatic portal vein, and hepatic vein, respectively. When hepatic glucose uptake surpasses glucose production, NHGB is negative, indicating glucose uptake. This methodology was extended to assess net hepatic balance for various substrates and hormones across the liver. Hepatic fractional glucose extraction was determined by dividing NHGB by HGL_{in}. Unidirectional HGU was computed as the product of HGL_{in} and the fractional extraction of [3-^{3}H]-glucose. NHGU and tracer-derived HGU produced comparable results. Non-HGU, attributed to primarily skeletal muscle glucose uptake, was derived by subtracting tracer-determined HGU from the glucose infusion rate (GIR) and correcting for glucose mass fluctuations over time. Direct glycogen synthesis, which quantifies glycogen formed directly from glucose taken up by the liver, was estimated by dividing the total radiolabeled glycogen stored in hepatic tissue by the specific activity of the glucose precursor pool. The radiolabeled glycogen measurement reflected total disintegrations per minute (dpm) of labeled glucose incorporated into glycogen, while the precursor pool's specific activity was determined as the ratio of labeled to unlabeled glucose (dpm/mg) in the inflowing blood, weighted by their contributions in the hepatic artery and portal vein. Net hepatic carbon retention (NHCR), representing total glycogen synthesis over time, was calculated using NHCR = NHGU + NHLU + NHAU + NHGlyU – HGO. Here, NHLU, NHAU, and NHGlyU denote the net hepatic uptake of

lactate, alanine, and glycerol, respectively, while HGO corresponds to hepatic glucose oxidation. Previous studies have validated hepatic glucose oxidation at approximately 0.2 mg/kg/min under various hyperinsulinemic-hyperglycemic clamp conditions, confirming its minimal contribution to total hepatic glucose flux (22; 23). Glycolytic flux was estimated as the sum of HGO and the net hepatic output of lactate, alanine, and glycerol. Finally, hepatic sinusoidal plasma insulin and glucagon concentrations were derived based on their relative contributions from the hepatic artery and portal vein, following established methodology.

Statistics

Data are expressed as mean ± SEM. A two-way repeated-measures ANOVA was conducted to evaluate differences between groups and changes over time in flux analyses, followed by Tukey's post hoc test for multiple comparisons. An unpaired two-tailed t-test was applied to analyze respective areas under the curve, while molecular analyses were assessed using a one-way ANOVA with Tukey's post hoc test. Normality checks were conducted for all statistical tests, confirming that the data followed a normal distribution. A significance threshold of $P < 0.05$ was applied. Statistical analyses were performed using GraphPad Prism software.

Results

AM Clamp Glucose and Hormone Data

During the AM clamp, euglycemia was successfully maintained in both groups **(Fig. 2A)**. Arterial and hepatic sinusoidal plasma insulin levels rose similarly in the Prime and the Prime + ↑GGN groups, reflecting the intended pattern based on AM insulin infusion rates (**Fig. 2B, C, Table 1).** The glucose infusion rate required to maintain euglycemia was comparable between groups (Prime: 9.5 ± 0.5 mg/kg/min vs. Prime + ↑GGN: 9.3 ± 1.3 mg/kg/min, **Fig. 2D**). As designed, arterial plasma glucagon levels remained basal in the Prime group (23 ± 1 pg/mL) but increased twofold in the Prime + ↑GGN group (49 ± 2 pg/mL), maintaining an equivalent yet elevated

insulin-to-glucagon ratio **(Fig. 2E).** This trend was mirrored in hepatic sinusoidal glucagon levels **(Fig. 2F, Table 1).** Despite the elevated glucagon levels in the Prime + ↑GGN group, net hepatic glucose balance during the AM clamp did not differ significantly between groups **(Fig. 2G)**.

PM Clamp Glucose and Hormone Data

Before the PM clamp, all animals returned to a state of hepatic glucose production. The PM clamp conditions mimicked a postprandial hormonal and substrate milieu by clamping plasma glucose at hyperglycemic levels using a combination of exogenous and portal vein glucose infusion **(Fig. 3A, B, D).** This created a negative arterial-to-portal vein glucose gradient to activate the portal glucose signal **(Fig. 3C)** while achieving a fourfold increase in insulin levels in both the arterial circulation and hepatic sinusoids **(Fig. 3E, F).** Arterial and hepatic sinusoidal plasma glucagon levels remained basal throughout the PM clamp in both groups **(Fig. 3G, H),** ensuring identical PM metabolic conditions.

The glucose infusion rate required to maintain hyperglycemia was similar between groups, averaging 13-13.5 mg/kg/min **(Fig. 4A, B).** Non-HGU did not differ significantly (Prime: 7.6 ± 0.9 mg/kg/min vs. Prime + ↑GGN: 9.4 ± 1.3 mg/kg/min, **Fig. 4C, D).** However, NHGU was 41% lower in the Prime + ↑GGN group (Prime: 6.1 ± 0.6 mg/kg/min vs. Prime + ↑GGN: 3.6 ± 0.4 mg/kg/min, **Fig. 4E, F).** The reduction in NHGU was not due to enhanced HGU, as HGU remained similar in both groups (Prime: 5.7 ± 0.5 mg/kg/min vs. Prime + ↑GGN: 5.2 ± 0.3 mg/kg/min**, Fig. 4G, H).** Instead, the Prime + ↑GGN group exhibited a twofold increase in hepatic glucose production compared to the Prime group, despite identical PM clamp conditions (Prime: −0.3 ± 0.3 mg/kg/min vs. Prime + ↑GGN: 1.7 ± 0.4 mg/kg/min, **Fig. 4I, J).** These data suggest that morning hepatic glucagon exposure has a prolonged impact on afternoon glucose metabolism, leading to sustained HGP.

During the PM clamp, arterial plasma NEFA levels and net hepatic NEFA uptake were equally suppressed in both groups **(Fig. 5A, B).** Blood glycerol and alanine levels also remained

constant with no group differences **(Fig. 5C–F).** However, arterial blood lactate levels and net hepatic lactate output were significantly reduced in the Prime + ↑GGN group compared to the Prime group (**Fig. 5G, H).** Despite increased PM HGP in the Prime + ↑GGN group, PM gluconeogenic flux to glucose-6-phosphate (G6P) was not significantly different between groups **(Fig. 6B),** suggesting that the increased HGP occurred upstream of G6P. However, PM glycolytic flux from G6P was reduced in the Prime + ↑GGN group, consistent with the observed lactate data **(Fig. 6C).** Additionally, the Prime + ↑GGN group exhibited reduced net glycogen flux **(Fig. 6D)** and a 53% decrease in direct glycogen synthesis (Prime: 3.2 ± 0.7 mg/kg/min vs. Prime + ↑GGN: 1.5 ± 0.2 mg/kg/min, **Fig. 6E).** Collectively, these findings demonstrate that prior morning glucagon exposure shifts hepatic glucose metabolism later in the day, promoting increased glucose production while impairing glycogen synthesis and glycolysis.

Discussion

The regulation of hepatic glucose metabolism is critical for maintaining glucose homeostasis throughout the day (24). An abundance of evidence suggests that breakfast plays a significant role in glycemic control and glycogen storage, influencing the metabolic response to subsequent meals in both healthy individuals as well as those with diabetes (2-4; 25). Meta-analyses have linked breakfast skipping to an increased risk of T2DM, suggesting that morning metabolic disturbances may have lasting effects (26; 27). Whether an elevation of glucagon is a contributor to the onset of diabetes is unclear (28-33). Nevertheless, the glucagon response to a mixed meal is generally greater in obese adults and those with T2DM than in normal weight, normoglycemic subjects (10; 34-36). Given that glucagon opposes insulin's anabolic actions by promoting glycogenolysis and HGP, understanding its role in postprandial metabolism is essential. Individuals with obesity and T2DM tend to exhibit exaggerated glucagon responses, further implicating its role in dysregulated glucose homeostasis.

The present study aimed to investigate the impact of morning hepatic glucagon exposure on hepatic glucose handling later in the day. Our findings indicate that prolonged morning glucagon exposure, even after glucagon levels return to normal, shifts hepatic metabolism in a manner that favors glucose release over storage during an afternoon HIHG clamp. Specifically, the Prime + ↑GGN group exhibited significantly increased HGP despite identical insulin and glucagon levels during the PM clamp, suggesting that prior glucagon exposure alters hepatic metabolic memory and impairs the liver's ability to incorporate glucose into glycogen efficiently. While HGU remained similar between groups, glycogen metabolism in the Prime + ↑GGN group was significantly impaired. This group exhibited reduced direct glycogen synthesis and net glycogen flux, implying that prior glucagon signaling established a metabolic state that prioritized glucose release over glycogen storage during a subsequent postprandial challenge.

Further evidence of this metabolic shift was observed in the reduction of glycolytic flux and lactate output in the Prime + ↑GGN group. Under normal physiological conditions, glucose entering the liver is directed toward glycolysis and/or glycogen synthesis. However, in this group, glucose was not efficiently utilized in these pathways, suggesting a diversion toward glycogenolysis and/or futile glucose cycling. Glycolysis, a crucial process for glucose metabolism, appeared to be downregulated in this group, supporting the notion that prolonged glucagon exposure can impair hepatic glucose processing. Interestingly, gluconeogenic flux did not differ between groups, indicating that increased HGP was not driven by enhanced gluconeogenesis but rather by alterations in glycogen metabolism, futile cycling, and/or impaired glycogenesis.

One potential mechanism for the increased HGP in the Prime + ↑GGN group is futile glucose cycling, in which glucose is phosphorylated to G6P and then reconverted to glucose, leading to inefficient glucose utilization. Another possibility is that morning glucagon exposure

promotes glycogen breakdown while impairing glycogen synthesis, maintaining an overall catabolic hepatic state. This effect may be mediated through sustained cAMP and PKA signaling, which are known to antagonize insulin's anabolic effects, thereby reinforcing a metabolic profile favoring glucose release rather than storage. These findings suggest that glucagon, beyond its immediate effects, leaves a lasting imprint on hepatic metabolism, potentially contributing to postprandial hyperglycemia later in the day.

The idea that morning hyperglucagonemia can have prolonged effects on hepatic glucose metabolism challenges the conventional view that glucagon's actions are purely acute. Instead, our findings suggest that morning glucagon exposure can establish a metabolic trajectory that persists even after glucagon levels normalize. This has important implications for individuals with obesity and T2DM, where dysregulated glucagon secretion may contribute to persistent impairments in glucose homeostasis. If morning hyperglucagonemia promotes an increased reliance on glucose release rather than storage, it may exacerbate postprandial hyperglycemia and impair long-term glycemic control.

In contrast, the Prime group, which did not receive elevated morning glucagon exposure, exhibited normal hepatic glucose handling (suppressed HGP), with insulin's effects dominating in the absence of glucagon-induced opposition. This group demonstrated efficient glycogen synthesis and glucose utilization, emphasizing the necessity of balanced glucagon-insulin signaling for optimal glucose metabolism.

In conclusion, our study demonstrates that morning glucagon exposure has a prolonged impact on hepatic glucose metabolism, shifting the liver's metabolic state toward sustained glucose release and impaired glycogen storage. The Prime + ↑GGN group exhibited increased HGP, reduced glycogen synthesis, and decreased glycolytic flux, indicating that prior glucagon signaling disrupted the liver's ability to efficiently handle glucose. These findings provide new

insights into the role of glucagon in regulating glucose homeostasis and suggest that elevated morning glucagon exposure may impair insulin's ability to prime the liver for enhanced glucose uptake and storage during subsequent meals. Given the relevance of glucagon dysregulation in metabolic disorders such as T2DM, future studies should further explore the molecular mechanisms underlying these effects and assess whether therapeutic strategies targeting glucagon signaling can improve metabolic outcomes.

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Author Contributions. H.W., D.S.E., and A.D.C. participated in the design of experiments; H.W. directed all experiments and collected data; B.F., K.Y., and T.H. participated in the experiments; B.F. and K.Y. were responsible for surgical preparation and oversight of animal care; H.W. and M.S.S. carried out biochemical and tissue analysis; H.W., D.S.E., and A.D.C. interpreted experimental results; H.W. prepared figures and drafted the manuscript; H.W., D.S.E., G.K., and A.D.C. edited and revised the manuscript; All authors approved the final version of the manuscript.

Guarantor Statement. A.D.C. is the guarantor of this work and, as such, has 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.

Conflict of Interest. A.D.C. has research contracts with Alnylam, Abvance, Fluidics, and Novo Nordisk, as well as grants from the NIH and the Helmsley Charitable Trust. A.D.C. is a consultant to Novo Nordisk, Paratus, Portal Insulin, Fractyl, and Thetis. No other individuals have conflicts of interest relevant to this article.

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	Group	
AM Clamp Parameter	**Prime (n=8)**	**Prime + ↑GGN (n=8)**
Arterial Plasma Insulin (µU/mL)	50 ± 4	51 ± 4
Hepatic Sinusoidal Plasma Insulin (µU/mL)	151 ± 6	170 ± 15
Hepatic Sinusoidal Plasma Glucagon (pg/mL)	34 ± 2	81 ± 3

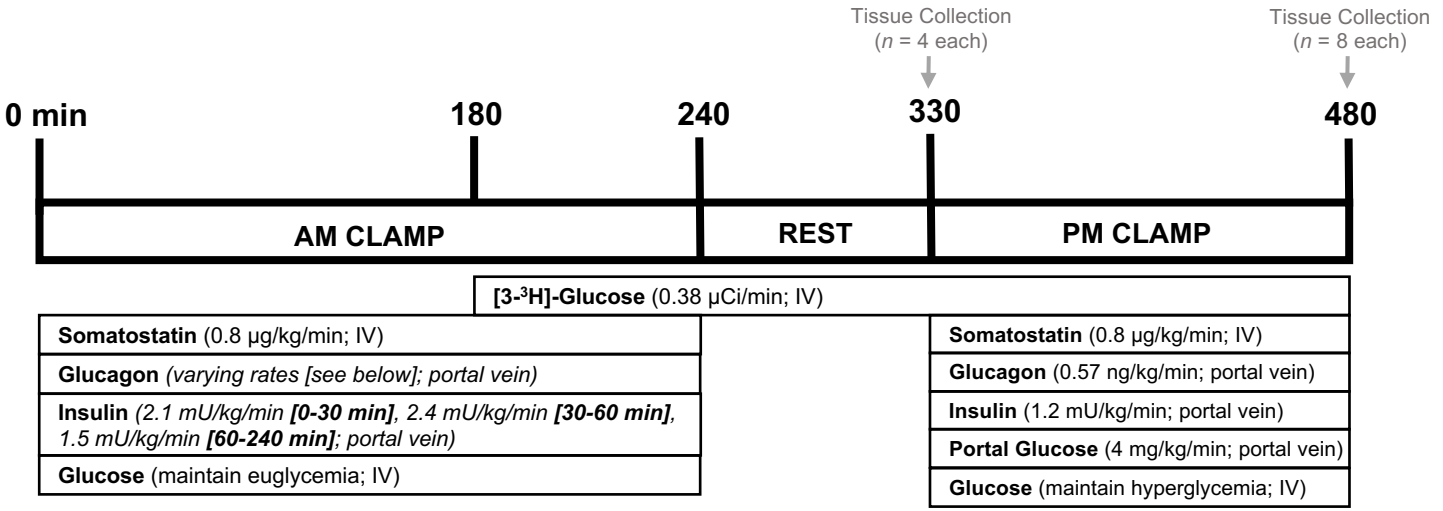
Figure Legends

1. **Figure 1: Experimental protocol.** Canines underwent a 4h euglycemic clamp in the morning (AM; 0 to 240 min). Somatostatin (SST) was infused to suppress the endogenous secretion of glucagon (GGN) and insulin. Insulin was replaced in a pattern that mimicked levels of insulin production previously observed during a duodenal glucose infusion. One group received basal glucagon (Prime) while the other received hyperglucagonemia (Prime + ↑GGN) in a pattern that maintained an equal molar ratio between insulin and glucagon throughout the AM clamp. Tracer infusion began near the end of the AM clamp (180 min) to allow enough time for equilibration before the start of the PM clamp. Following the AM clamp, there was a 1.5h rest period (240-330 min) where all infusions were halted. At the end of this rest period, dogs from each group were euthanized, and hepatic tissue was collected for molecular analysis (*n*=4/group). The remainder of the dogs (*n*=8/group) underwent a 2.5h PM hyperinsulinemic-

hyperglycemic clamp with portal glucose delivery (330 to 480 min). Tissue collection for these dogs occurred at the end of the PM clamp. Details can be found in the methods section.

2. **Figure 2: Morning (AM) clamp glucose and hormone flux data.** Arterial plasma glucose (A), arterial plasma insulin (B), hepatic sinusoidal plasma insulin (C), glucose infusion rate (D), arterial plasma glucagon (E), hepatic sinusoidal plasma glucagon (F), and net hepatic glucose balance (G) are shown for the Prime and the Prime + ↑GGN groups; *n*=8/group, throughout the AM clamp. Data are expressed as mean ± SEM. ***P<0.001, ****P<0.0001 between groups. ns = non-significant.
3. **Figure 3: Afternoon (PM) clamp glucose and hormone flux data.** A vertical line at 330 min separates the resting period from the onset of the PM clamp. Arterial plasma glucose (A), portal vein plasma glucose (B), the difference between the artery and the portal vein plasma glucose levels (C), hepatic glucose load (D), arterial plasma insulin (E), hepatic sinusoidal plasma insulin (F), arterial plasma glucagon (G), and hepatic sinusoidal plasma glucagon (H) are shown throughout the 2.5h hyperinsulinemic-hyperglycemic PM clamp period in both the Prime and Prime + ↑GGN groups; *n*=8/group. There were no differences between the two groups for any of the parameters measured. Data are expressed as mean ± SEM. ns = non-significant.
4. **Figure 4: Glucose uptake and storage during the PM HIHG clamp.** A vertical line at 330 min separates the resting period from the onset of the PM clamp. Glucose infusion rate (A), non-hepatic glucose uptake (C), net hepatic glucose uptake (E), tracer-determined hepatic glucose uptake (G), and hepatic glucose production (H) are shown over time for the Prime and Prime + ↑GGN groups, *n*=8/group. The respective AUCs for each parameter are shown for both groups (B, D, F, H, J). Data are expressed as mean ± SEM. *P<0.05, **P<0.01 between groups. ns = non-significant.

5. **Figure 5: PM clamp fatty acid and metabolite flux data.** A vertical line at 330 min separates the resting period from the onset of the PM clamp. Arterial plasma non-esterified fatty acids (NEFA) (A), net hepatic NEFA uptake (B), arterial blood glycerol (C), net hepatic glycerol uptake (D), arterial blood alanine (E), net hepatic alanine uptake (F), arterial blood lactate (G), and net hepatic lactate uptake (H) are shown throughout the PM HIHG clamp for the Prime and Prime + ↑GGN groups, *n*=8/group. Data are expressed as mean ± SEM. *P<0.05, **P<0.01 between groups. ns = non-significant.
6. **Figure 6: Glucose flux and glycogen data for the PM clamp.** A vertical line at 330 min separates the resting period from the onset of the PM clamp. Net gluconeogenic/glycolytic flux (A), gluconeogenic flux (B), glycolytic flux (C), net glycogen flux (D), and direct glycogen synthesis from glucose (E) are shown throughout the PM clamp for the Prime and Prime + ↑GGN groups, *n*=8/group. Data are expressed as mean ± SEM. *P<0.5, **P<0.01 between groups. ns = non-significant.

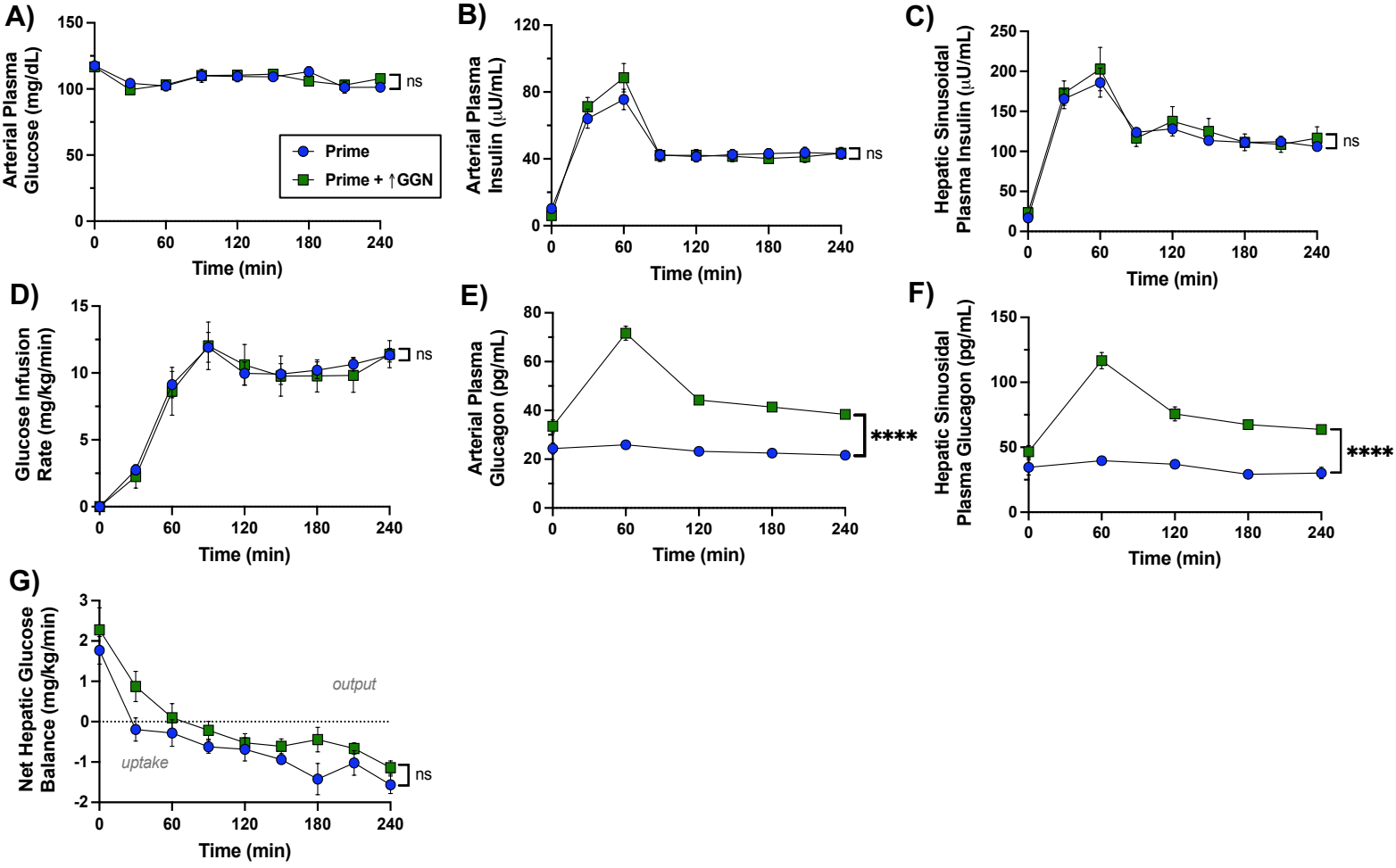


AM CLAMP GLUCAGON RATES (ng/kg/min):

Prime (Basal glucagon, *n=8*):
0-240 min: 0.57

Prime + ↑GGN (Hyperglucagonemia, *n=8*):
0-30 min: 2.28
30-60 min: 2.60
60-240 min: 1.63

Figure 1: Experimental protocol. Canines underwent a 4h euglycemic clamp in the morning (AM; 0 to 240 min). Somatostatin (SST) was infused to suppress the endogenous secretion of glucagon (GGN) and insulin. Insulin was replaced in a pattern that mimicked levels of insulin production previously observed during a duodenal glucose infusion. One group received basal glucagon (Prime) while the other received hyperglucagonemia (Prime + ↑GGN) in a pattern that maintained an equal molar ratio between insulin and glucagon throughout the AM clamp. Tracer infusion began near the end of the AM clamp (180 min) to allow enough time for equilibration before the start of the PM clamp. Following the AM clamp, there was a 1.5h rest period (240-330 min) where all infusions were halted. At the end of this rest period, dogs from each group were euthanized, and hepatic tissue was collected for molecular analysis (*n*=4/group). The remainder of the dogs (*n*=8/group) underwent a 2.5h PM hyperinsulinemic-hyperglycemic clamp with portal glucose delivery (330 to 480 min). Tissue collection for these dogs occurred at the end of the PM clamp. Details can be found in the methods section.



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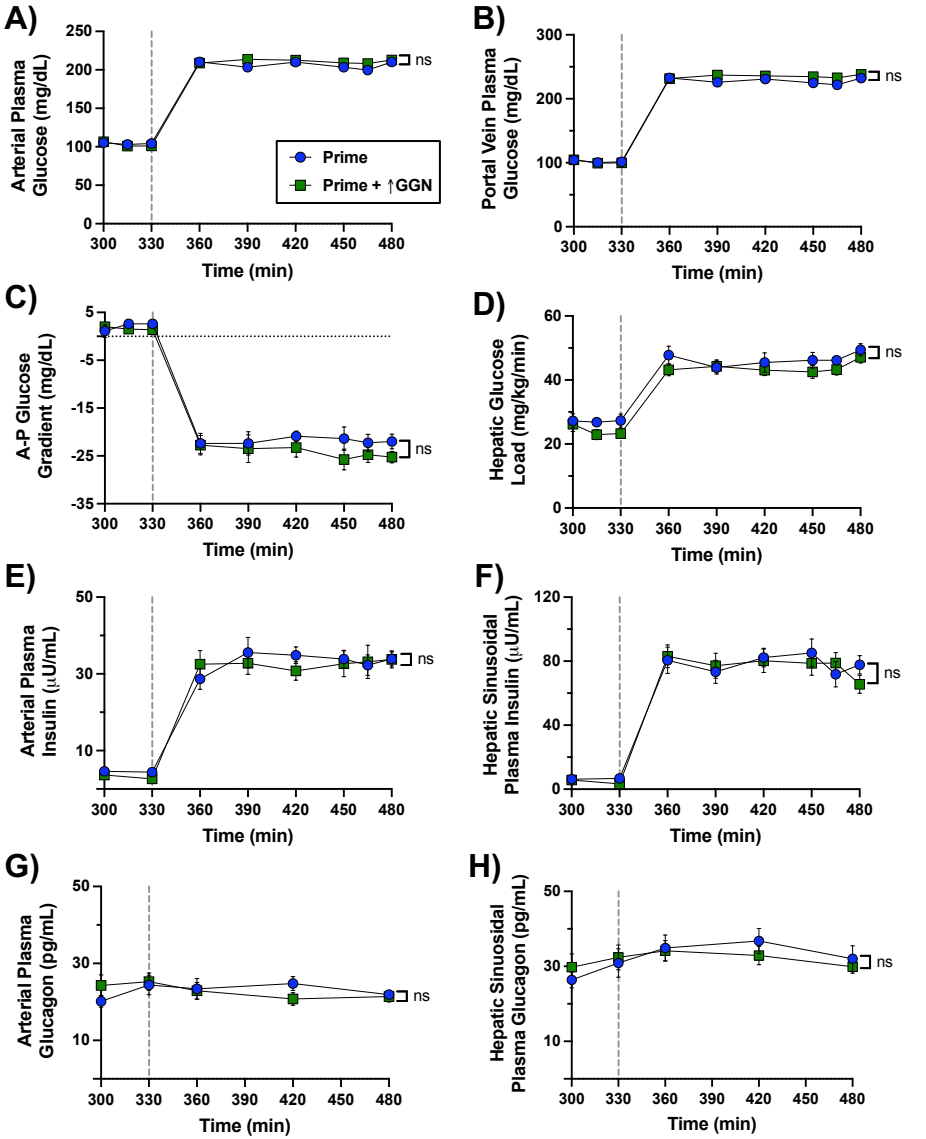

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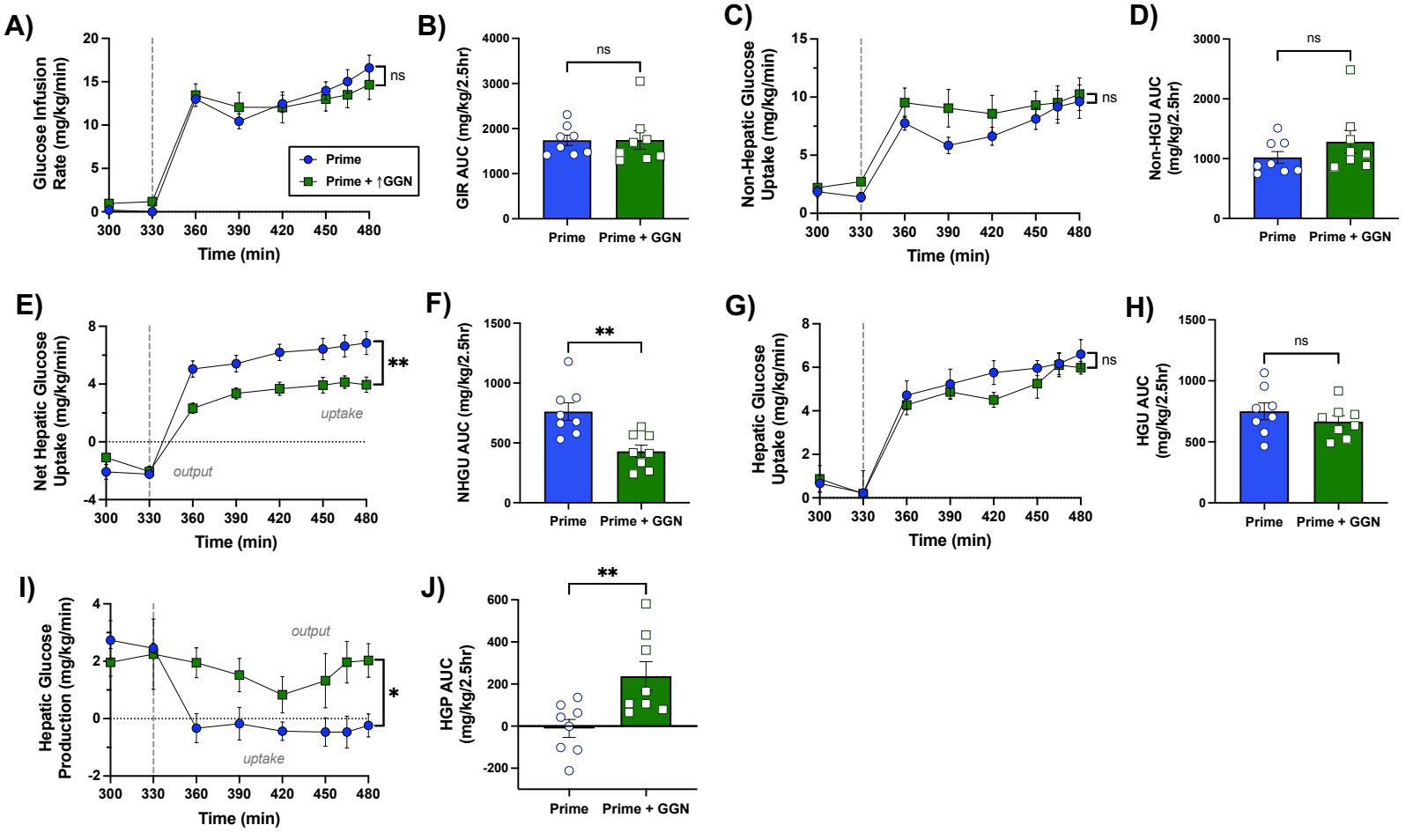

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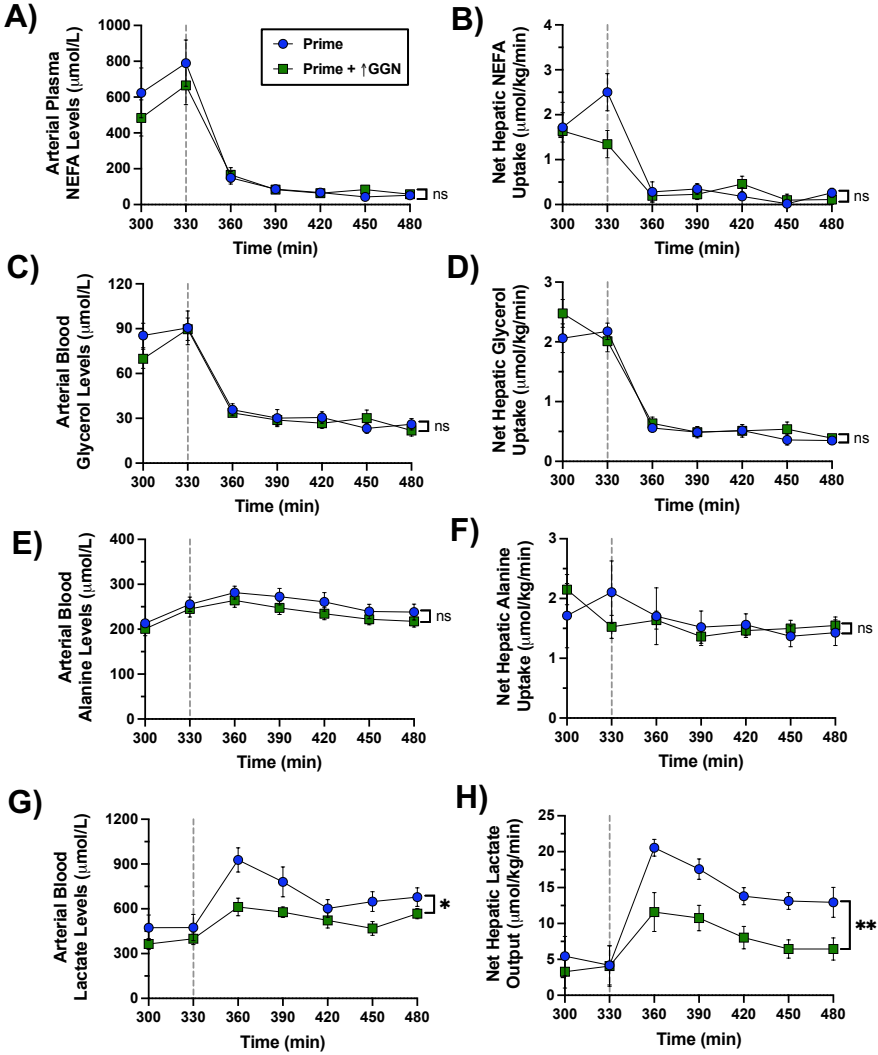

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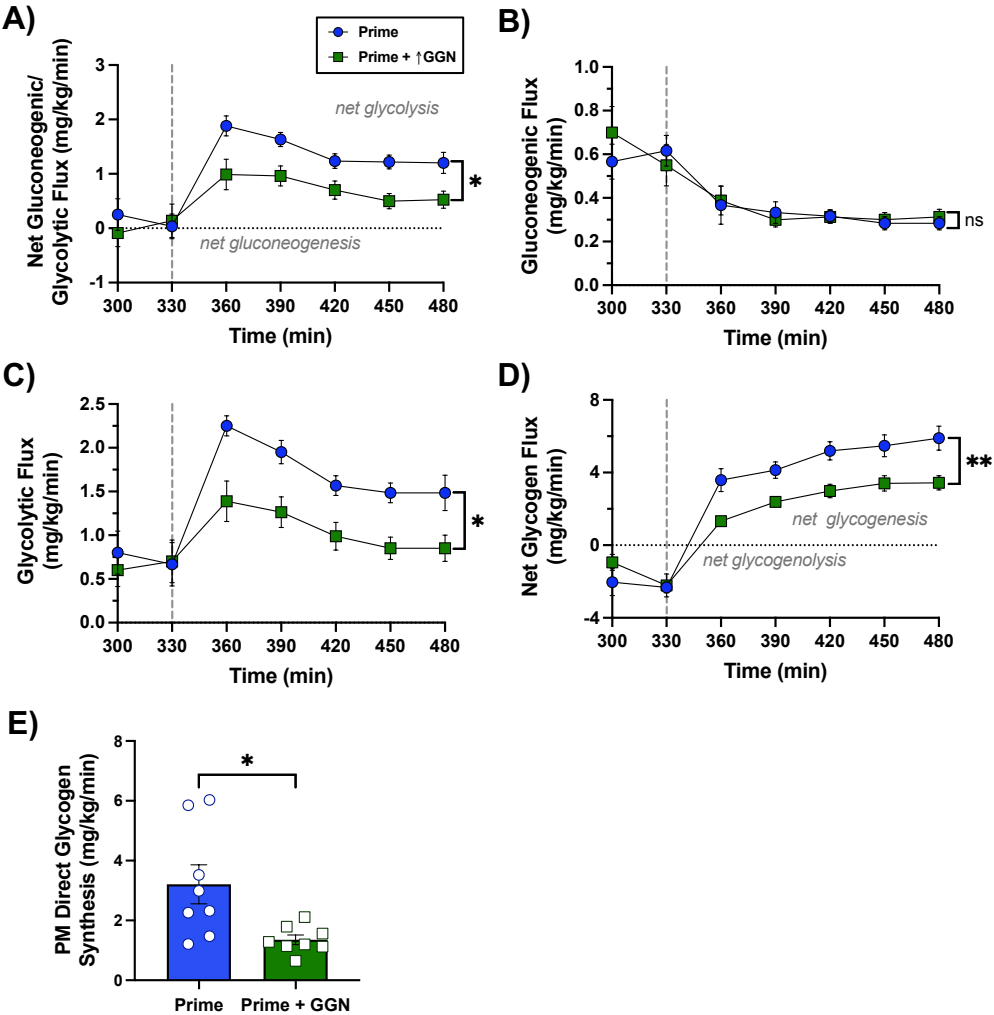

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