Morning Elevation in Insulin Enhances Afternoon Postprandial Insulin Action and Glucose Effectiveness

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Abstract

The second meal phenomenon refers to the improved glycemic response observed after consuming a second identical meal. It is well known that postprandial hepatic glucose uptake (HGU) is determined by the combined effects of three regulatory factors: insulin action (hyperinsulinemia; HI), glucose effectiveness (hyperglycemia; GE), and glucose delivery into the hepatoportal circulation (portal glucose signal; PGS). While our previous studies demonstrated the importance of morning (AM) hyperinsulinemia in priming the liver for increased HGU later in the day, the question remains as to which aspect of the afternoon (PM) response is enhanced by morning insulin. Thus, to provide insight into the underlying mechanism of the 2nd meal effect, we sought to determine the extent to which PM HI, GE, and the PGS are impacted by AM hyperinsulinemia. Dogs underwent an AM clamp with either a 4h hyperinsulinemic prime (Prime, *n=8*) or basal insulin delivery (No Prime, *n=8*). After a 1.5h non-clamp period, both groups were challenged with a PM hyperglycemic clamp (with the PGS) in the presence of basal insulin. During the PM clamp, net HGU was significantly elevated in Prime vs. No Prime group (mean of 2.2±0.3 vs. 0.1±0.3 mg/kg/min, respectively, $P<0.005$), indicating an enhancement of GE and/or the PGS. There was no difference in PM non-HGU (4.5±0.3 vs. 4.0±0.3 mg/kg/min). In previous experiments, when all three factors (HI, GE, PGS) were present in the PM, net HGU was 6.3±1.7 vs. 2.4±1.1 mg/kg/min in groups receiving an AM insulin prime vs. basal insulin, respectively. Therefore, the AM insulin prime enhanced the effects of HI, GE, and the PGS on PM HGU by 3.9 mg/kg/min, whereas it enhanced the effects of glucose (GE and the PGS), in the absence of a rise in insulin, by 2.1 mg/kg/min. Taken together, the data show that AM HI enhanced PM insulin action and GE (including the PGS) to an equal extent. This suggests that AM insulin priming enhances cellular targets common to both insulin and glucose signaling in the PM.

Introduction

Postprandial glucose regulation is essential for maintaining metabolic homeostasis, as impaired glucose disposal is a hallmark of insulin resistance and type 2 diabetes (1; 2). A well-characterized but not fully understood phenomenon in glucose metabolism is the 2nd meal effect, where the glycemic response to a second, identical meal is improved compared to the first (3-5). This adaptive response is linked to changes in liver glucose metabolism, specifically hepatic glucose uptake (HGU), which plays a crucial role in regulating postprandial glucose excursions (6). While the general improvement in HGU following the second meal is recognized, the precise mechanisms underlying how prior insulin exposure enhances subsequent HGU remain to be elucidated.

The regulation of postprandial HGU is influenced by the interaction of three primary factors: insulin action (hyperinsulinemia; HI), glucose effectiveness (hyperglycemia; GE), and the portal glucose signal (PGS) (7; 8). In this context, hyperinsulinemia refers to the physiological rise in insulin levels following a meal, which promotes insulin-dependent glucose uptake in the liver and peripheral tissues (9). Insulin's action on the liver helps regulate both glucose production and storage, and its effects on HGU are key in postprandial glucose clearance, even equaling muscle (10). Glucose effectiveness, on the other hand, describes the ability of glucose itself to enhance its own uptake and storage, independent of insulin (11). This insulin-independent effect contributes significantly to glucose homeostasis, especially when insulin action is compromised (12; 13). The portal glucose signal is the third factor, referring to the neural response that occurs when glucose is absorbed or delivered directly into the hepatoportal circulation (7). This signal plays a crucial role in coordinating the liver's response to changes in glucose levels, ensuring efficient glucose utilization (14-16).

In our previous work, we demonstrated that morning (AM) hyperinsulinemia primes the liver for an enhanced HGU response to a hyperinsulinemic-hyperglycemic (HIHG) clamp later in the day (17). However, the specific mechanisms driving the augmented HGU observed in the afternoon (PM) response, particularly in the context of the 2nd meal phenomenon, are not fully understood. While AM hyperinsulinemia has been shown to enhance the liver's response to the full combination of factors that regulate HGU (HI, GE, and PGS), it is unclear which of these factors is responsible for the enhanced HGU observed during a second meal.

We hypothesize that AM hyperinsulinemia will enhance PM glucose uptake through both insulin-dependent and insulin-independent mechanisms, indicating that hepatic metabolic pathways common to both insulin action and glucose effectiveness are primed by prior insulin exposure for subsequent HGU. By dissecting the contributions of insulin action, glucose effectiveness, and the portal glucose signal, this study will provide new mechanistic insights into the temporal dynamics of hepatic glucose metabolism. Ultimately, this research will expand our understanding of how metabolic conditions established in the morning influences subsequent postprandial responses and could have significant therapeutic implications for improving glucose control, particularly in individuals with glucose intolerance or type 2 diabetes.

Research Design and Methods

Animal care and surgical procedures

16 adult mongrel dogs (8 males, 8 females) with an average body weight of 27.6 ± 1.0 kg were used in these experiments. The animals were obtained from a USDA-approved vendor and housed according to the standards set by the American Association for the Accreditation of Laboratory Animal Care. All procedures were reviewed and approved by the Vanderbilt Institutional Animal Care and Use Committee. Two weeks before the experimental procedures, the animals underwent a laparotomy under general anesthesia for the surgical placement of

blood flow probes around the hepatic artery and hepatic portal vein. Additionally, catheters were implanted for blood sampling in the hepatic vein, hepatic portal vein, and femoral artery. Infusion catheters were positioned in the splenic and jejunal veins, which supply the hepatic portal vein, as well as the inferior vena cava. These catheters were secured subcutaneously until the day of the experiment, when they were exteriorized. The dogs were fed a controlled diet consisting of chow and meat, with a macronutrient composition of 46% carbohydrates, 34% protein, 14.5% fat, and 5.5% fiber. Before the experiment, the animals underwent an 18-hour fasting period. Assessments were conducted to ensure the dogs were healthy before the experiment, requiring that the dogs had consumed at least 75% of their most recent meal, maintained a leukocyte count below 18,000/mm^3, and had a hematocrit level above 34%. Blood collection was limited to no more than 20% of the animal's total blood volume over the course of a day.

Experimental Design

The experimental protocol lasted 8 hours and included two glucose clamp phases, separated by a rest period. These clamps were designed to simulate postprandial glucose dynamics at times corresponding to morning and midday meals. The first phase, referred to as the AM clamp, occurred from 0 to 240 minutes, followed by a break before the PM clamp, which took place from 330 to 480 minutes **(Fig. 1).** Blood samples were collected every 15 to 30 minutes from the femoral artery, hepatic portal vein, and hepatic vein to measure hormone and metabolite levels. Arterial plasma glucose was assessed every five minutes, and exogenous glucose infusion into a peripheral vein was adjusted as needed to maintain target glucose concentrations.

Morning (AM) Clamp Period (0-240 min) and Non-Clamp Period (240 – 330 min)

To suppress endogenous pancreatic insulin and glucagon secretion, somatostatin (Bachem, Torrance, CA) was infused into the inferior vena cava (0.8 µg/kg/min) at the onset of

the AM clamp. Glucagon (GlucaGen, Boehringer Ingelheim, Ridgefield, CT) was replaced intraportally at basal levels (0.57 ng/kg/min). One of the experimental groups, Prime, received morning insulin (Novolin R; Novo Nordisk, Basværd, Denmark) infusion designed to replicate the physiologic insulin secretion pattern previously observed following a morning duodenal glucose infusion (replaced 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) (17). The other group, No Prime, received intraportal basal insulin replacement in the morning (0.25 mU/kg/min). Prior research has shown that the enhancement of HGU in the afternoon is driven by an increase in insulin during the morning, rather than a rise in morning glucose levels (17). Therefore, glucose was clamped at euglycemia (~100 mg/dL plasma glucose) for the entirety of the AM clamp. To allow for tracer-determined 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. After the AM clamp (240 min), all infusions, except for the tracer, were discontinued for 1.5h (non-clamp period). To characterize the hepatic metabolic state prior to the initiation of the PM clamp, blood samples were obtained from the femoral artery, portal vein, and hepatic vein at 300, 315, and 330 minutes **(Fig. 1).**

Afternoon (PM) Clamp Period (330 – 480 min)

Both the Prime and No Prime groups underwent an identical 2.5-h hyperglycemic clamp with basal insulin and glucagon during the afternoon phase **(Fig. 1).** This protocol was designed to isolate the effects of the morning insulin prime on PM insulin action vs. glucose and the portal glucose signal. Thus, basal insulin levels were maintained throughout the PM clamp. To achieve this, somatostatin was continuously infused via a peripheral vein (0.8 µg/kg/min), while basal glucagon (0.57 ng/kg/min) and fourfold basal insulin (1.2 mU/kg/min) were delivered intraportally. To stimulate the portal glucose signal, glucose was directly infused into the hepatic portal vein at a rate of 4 mg/kg/min. Additionally, a primed, continuous glucose infusion into a

peripheral vein was administered to rapidly elevate plasma glucose levels to ~200 mg/dL, maintaining hyperglycemia for the duration of the PM clamp. These conditions were intended to mimic the postprandial metabolic state seen following carbohydrate ingestion (7). To further investigate the liver's metabolic response, all dogs were anesthetized with infusions still ongoing. At the end of the PM clamp (480 min) hepatic tissue was rapidly excised, flash-frozen in liquid nitrogen, and stored at -80°C for subsequent molecular analyses, following which the animals were euthanized.

Analyses

Biochemical and Molecular Methods

To evaluate hormone and substrate flux across the liver, whole blood samples were collected and analyzed thereby permitting the use of arteriovenous balance techniques. Plasma glucose levels were measured in quadruplicate with an Analox GM9 glucose analyzer throughout the experiment. The concentrations of insulin (PI-12K, MilliporeSigma, Burlington, MA), glucagon (GL-32K, MilliporeSigma), and cortisol (VUMC Analytical Services Core in-house primary antibody with I^{125} cortisol from MP Biomedicals, Santa Ana, CA) were quantified using radioimmunoassay (18). Key metabolic intermediates, including lactate, glycerol, alanine, and non-esterified fatty acids (NEFA), were assessed via enzymatic spectrophotometric assays. To measure the specific activity of [3-^{3}H]-glucose, plasma samples were deproteinized using the Somogyi method, followed by liquid scintillation counting (19; 20). The terminal hepatic glycogen content was quantified using the Keppler and Decker amyloglucosidase method (21).

At the end of the PM clamp, hepatic tissue was collected for qPCR analysis, which has previously been optimized for canine-specific targets using validated primers (18). Gene expression levels were normalized to GAPDH. Additionally, baseline liver samples from five dogs that were fasted overnight were included for comparison.

Calculations

Arterio-venous difference calculations were used to determine net hepatic glucose balance (NHGB) using the following equation: NHGB = $[HGL_{out}] - [HGL_{in}]$. Glucose output from the liver (HGL_{out}) was calculated as $[HGL_{out}] = [BF_h \times G_h]$, while glucose coming into the liver was calculated as $[HGL_{in}] = [(BF_a \times G_a) + (BF_p \times G_p)]$. In these equations, G represents the concentration of blood glucose, BF indicates measured blood flow, and A, P, and H correspond to the hepatic artery, hepatic portal vein, and hepatic vein, respectively. Negative values of NHGB indicate that hepatic glucose uptake exceeds glucose production, suggesting net glucose uptake. This approach was also applied to assess net hepatic balance for other substrates and hormones. To calculate hepatic fractional glucose extraction, NHGB was divided by HGLin. Unidirectional hepatic glucose uptake (HGU) was determined by multiplying HGLin by the fractional glucose extraction of [3-^{3}H]-glucose. Non-HGU, which primarily represents glucose uptake by skeletal muscle, was calculated by subtracting tracer-derived HGU from the glucose infusion rate (GIR) and adjusting for changes in glucose mass over time. Direct hepatic glycogen synthesis was estimated by dividing the total radiolabeled glycogen stored in the liver by the specific activity of the glucose precursor pool. This was measured in disintegrations per minute (dpm) of labeled glucose incorporated into glycogen. The specific activity of the glucose precursor pool was determined by the ratio of labeled to unlabeled glucose (dpm/mg) in the inflowing blood, considering contributions from both the hepatic artery and portal vein. To calculate net hepatic carbon retention (NHCR), which reflects total glycogen synthesis over time, the equation NHCR = NHGU + NHLU + 2(NHAU) + NHGlyU – HGO was used. Here, NHLU, NHAU, and NHGlyU represent net hepatic uptake of lactate, alanine, and glycerol, respectively, and HGO refers to hepatic glucose oxidation. NHAU was doubled to account for net hepatic uptake of all amino acids, as alanine accounts for approximately 50% of

gluconeogenic amino acid precursor uptake (22; 23). Previous studies have shown that hepatic glucose oxidation is minimal, approximately 0.2 mg/kg/min under hyperinsulinemic-hyperglycemic clamp conditions, thus contributing little to overall hepatic glucose flux (24; 25). Glycolytic flux was estimated by adding HGO and the net hepatic output of lactate, alanine, and glycerol. Finally, plasma concentrations of insulin and glucagon in the hepatic sinusoidal space were derived based on the relative contributions of the hepatic artery and portal vein, as previously established.

Statistics

Results are expressed as mean ± SEM. Group differences and temporal changes in flux analyses were evaluated using a two-way repeated-measures ANOVA, followed by Tukey's post hoc test for multiple comparisons. Areas under the curve were compared using an unpaired two-tailed t-test, while molecular outcomes were analyzed via one-way ANOVA with Tukey's post hoc correction. Data normality was confirmed before statistical testing. A threshold of $P < 0.05$ was used to define statistical significance. All analyses were performed using GraphPad Prism software.

Results

AM Clamp Glucose and Hormone Data

Plasma glucose levels were clamped at euglycemia (105-115 mg/dL) in both groups during the morning clamp **(Fig. 2A)**. Arterial plasma insulin was maintained at basal levels in the No Prime group (mean of 6 ± 1 μU/mL), whereas in the Prime group, insulin levels were elevated as intended (mean of 32 ± 3 μU/mL) **(Fig. 2B, C).** This pattern was reflected in the hepatic sinusoidal plasma insulin levels, with insulin levels in the Prime group being nearly 6-fold of that in the No Prime group **(Fig. 2D, E, Table 1).** Arterial and hepatic sinusoidal plasma

glucagon levels were matched between the two groups **(Table 1).** The exogenous glucose delivered to adequately clamp plasma glucose levels was significantly greater in the Prime group compared to the No Prime group, as expected **(Fig. 2F, G, Table 1).** In summary, the Prime group received elevated amounts of insulin (and subsequently glucose to maintain euglycemia) in the hepatic sinusoids and arterial circulation during the AM clamp, representative of a morning insulin prime, whereas the No Prime group received basal levels of insulin, representative of an extended fast.

PM Clamp Glucose, Hormone, and Metabolite Data

Both groups were subject to identical afternoon conditions, which consisted of clamping glucose levels at hyperglycemia (230-240 mg/dL, **Fig. 3A, B)**, activating the portal glucose signal by delivering some glucose into the hepatic portal vein to establish a negative arterial-to-portal glucose gradient (**Fig. 3C**) and maintaining arterial and hepatic sinusoidal plasma insulin at basal levels **(Table 1, Fig. 3D).** Plasma glucagon levels were replaced at basal levels **(Table 1).** Despite having matched parameters during the PM clamp, the Prime group required 37% more glucose to maintain hyperglycemia (mean GIR of 7.0 $\pm$ 0.4 vs. 4.6 $\pm$ 0.4 mg/kg/min in Prime vs. No Prime, respectively, **Fig. 4A, B**). This increase in the GIR in the Prime group was not attributed to non-hepatic glucose uptake, which was not different between the two groups (mean of 4.5 $\pm$ 0.3 mg/kg/min in Prime vs. mean of 4.0 $\pm$ 0.3 mg/kg/min in No Prime, **Fig. 4C, D**). Rather, it was due to a substantial increase in PM net hepatic glucose uptake in the Prime group (mean of 2.2 $\pm$ 0.3 vs. 0.1 $\pm$ 0.3 mg/kg/min in Prime vs. No Prime, respectively, **Fig. 4E, F**). The enhancement in net hepatic glucose uptake in the Prime group compared to the No Prime group was attributed to an increase in net glycolytic flux **(Fig. 4G)** and net glycogenesis **(Fig. 4H)**. Thus, these data show that a morning insulin prime has lasting effects on afternoon hepatic glucose handling, resulting in increased afternoon net hepatic glucose uptake despite the presence of basal insulin during the PM clamp.

When comparing plasma non-esterified fatty acid (NEFA) levels, arterial NEFA levels were significantly greater in the No Prime group vs. the Prime group, although net hepatic NEFA uptake was similar between the two groups **(Fig. 5A, B)**. It is important to note that arterial NEFA levels were not at the same level at the onset of the PM clamp, likely due to the lingering effects of the morning insulin prime on suppressing lipolysis in the Prime group. Additionally, arterial blood glycerol and net hepatic glycerol uptake were not significantly different between groups **(Fig. 5C, D)** suggesting that, despite the elevated arterial NEFA levels in the No Prime group, hepatic processing of NEFAs and glycerol was not impaired. This may indicate that the excess circulating NEFAs were diverted toward re-esterification in peripheral tissues rather than being taken up and oxidized by the liver, although this pathway likely contributes only minimally. As for alanine, while arterial blood levels were not different between groups, the Prime group had reduced net hepatic alanine uptake compared to the No Prime group **(Fig. 5E, F).** Lastly, arterial blood lactate levels were similar between groups, but the Prime group had 5-fold greater net hepatic lactate output than the No Prime group, which is consistent with the Prime group exhibiting net glycolysis **(Fig. 5G, H).**

Glycogen Data and Terminal Hepatic Tissue Analyses

The amount of tritiated glucose incorporated into hepatic glycogen stores during the PM clamp, denoted as direct glycogen synthesis, was comparable between groups **(Fig. 6A)**. However, terminal hepatic glycogen was greater in the Prime group compared to the No Prime group (mean of 43 $\pm$ 3 vs. 36 $\pm$ 1 mg glycogen/g liver in Prime vs. No Prime, respectively, **Fig. 6B)**. Upon assessing hepatic gene expression at the end of the PM clamp, compared to baseline, transcript levels of phosphoenolpyruvate carboxykinase 1 (PCK1; **Fig. 6C**), glucose-6-phosphatase catalytic subunit 1 (G6PC1, **Fig. 6D**), and liver-specific glycogen phosphorylase (PYGL) mRNA **(Fig. 6E)** were reduced similarly between the two groups. On the other hand, glucokinase (GCK, **Fig. 6F**), sterol regulatory element binding protein-1c (SREBP-1c, **Fig. 6G**),

and fatty acids synthase (FAS) gene expression (**Fig. 6H)** at the end of the PM clamp were significantly elevated in the Prime group compared to the No Prime group. Acetyl-coA carboxylase (ACC) mRNA was not significantly different between groups (data not shown). These data further support the notion that morning insulin exposure has a lasting effect on the transcriptional landscape of the liver, allowing for enhanced PM glucose handling.

Discussion

The second meal phenomenon, characterized by an improved glycemic response to a second, identical meal, has recently gained increasing attention due to its potential therapeutic implication in managing postprandial glucose levels, particularly in individuals with insulin resistance and type 2 diabetes (3; 5; 26-30). Our previous research established that a morning elevation in insulin primes the liver for enhanced HGU in response to a subsequent glucose load (17; 31). However, the precise mechanisms underlying this improvement during PM hepatic glucose handling have remained unclear. In the present study, we identify the key mechanisms responsible for the augmented HGU observed in the afternoon following AM insulin priming, uncovering both insulin-dependent and insulin-independent components that contribute to enhanced PM glucose metabolism. Specifically, we demonstrate that AM hyperinsulinemia amplifies not only insulin action in the afternoon, but also glucose effectiveness and/or the portal glucose signal, leading to a coordinated increase in net HGU. These findings highlight the complex interplay between these signals in modulating postprandial HGU and, for the first time, provide novel mechanistic insight into how a morning insulin prime elicits its effects on the liver to improve glucose handling with subsequent glucose loads.

To assess the impact of AM insulin priming on PM net hepatic glucose uptake, we previously performed a series of control experiments in which dogs underwent a PM HIHG clamp following either morning basal insulin or elevated insulin delivery **(Fig. 7A).** The priming

effect of AM insulin was quantified as the difference in PM NHGU between these two conditions, which was 3.8 mg/kg/min. In the current study, dogs underwent a PM hyperglycemic clamp with basal insulin to isolate the effects of glucose (including both glucose effectiveness and the activation of the portal glucose signal) from the effects of insulin **(Fig. 7B).** Consistent with prior findings, the AM insulin prime significantly enhanced PM NHGU, with the difference between the No Prime and Prime groups, amounting to 2.1 mg/kg/min. This represents the contribution of glucose effectiveness and the portal glucose signal to the priming effect, independent of elevated PM insulin levels. Comparing the findings from both studies, AM insulin priming enhanced PM NHGU by 3.8 mg/kg/min when PM insulin was elevated versus by 2.1 mg/kg/min when insulin remained at basal levels, indicating that approximately 45% of the priming effect was attributable to increased PM insulin action **(Fig. 7C).** Insulin-primed animals exhibited significantly greater PM net HGU, indicating increased hepatic sensitivity to glucose itself. Thus, AM insulin priming had a similar impact on PM insulin action and glucose action, suggesting that priming influences cellular mechanisms common to both insulin and glucose signaling. These findings provide strong evidence that prior insulin exposure causes the liver to be conditioned to respond more effectively to both insulin and glucose in the PM, optimizing subsequent hepatic glucose disposal.

To understand how AM insulin priming enhances hepatic responsiveness to glucose independently of insulin, we examined hepatic intermediary metabolism under basal PM insulin conditions. Beyond improved PM net HGU, AM insulin priming altered downstream metabolism, with primed animals showing elevated PM net glycolytic flux and higher terminal hepatic glycogen content, despite similar rates of direct glycogen synthesis from tritiated glucose. This suggests enhanced glucose cycling in the Prime group, likely via increased indirect glycogen synthesis or accelerated glucose-6-phosphate turnover, facilitating more efficient glucose storage. These results demonstrate that prior insulin exposure metabolically conditioned the

liver to utilize glucose more effectively in the PM without requiring elevated insulin. This flexible metabolic phenotype is further supported by a fivefold increase in net hepatic lactate output in the Prime group, consistent with increased glycolytic throughput and possibly enhanced pyruvate cycling. While elevated lactate in the Prime group may reflect glycolytic flux surpassing immediate oxidative demands, the concurrent rises in glycogen content and glucokinase mRNA expression suggest coordinated activation of multiple glucose disposal pathways during the PM clamp.

To explore the molecular basis for the enhancement of HGU following AM insulin priming, we assessed hepatic gene expression at the end of the PM clamp. Compared to animals that received basal insulin in the morning (No Prime), those that received an AM insulin prime (Prime) exhibited elevated expression of GCK, SREBP-1c, and FAS, consistent with increased activation of insulin-responsive and lipogenic transcriptional programs (32; 33). Interestingly, ACC expression did not differ between groups, suggesting a selective induction of SREBP-1c targets or potential post-transcriptional regulation limiting ACC expression despite transcriptional priming (34). Meanwhile, gluconeogenic and glycogenolytic genes such as PCK1 and PYGL were similarly suppressed in both groups when compared to basal, indicating that the presence of elevated glucose alone is sufficient to downregulate these genes and that this effect is independent of prior insulin exposure. These findings support the concept that AM insulin primes the liver by selectively enhancing glucose uptake and lipid synthesis pathways while suppressing opposing catabolic processes. This coordinated shift in gene expression establishes a permissive transcriptional environment that poises the liver to more effectively dispose of glucose during the postprandial period.

In addition to changes in glucose metabolism, the PM clamp data revealed important differences in hepatic and systemic substrate handling that further characterize the metabolic adaptations of enhanced PM glucose action induced by AM insulin priming. During the PM

clamp, arterial NEFA levels were significantly elevated in the No Prime group compared to the Prime group, yet net hepatic NEFA uptake remained similar between groups. This dissociation indicates that hepatic fatty acid metabolism was not a primary factor driving the enhanced glucose disposal observed in the primed liver. Similarly, glycerol fluxes were comparable between groups, suggesting hepatic lipid processing was largely unaffected. Notably, the No Prime group exhibited increased net hepatic alanine uptake despite similar arterial alanine concentrations, potentially reflecting a compensatory increase in amino acid catabolism due to impaired glucose handling, although gluconeogenesis was not different between groups. These substrate flux patterns reinforce the concept that AM insulin priming shifts hepatic metabolism to preferentially utilize glucose, promoting more efficient energy partitioning and metabolic flexibility during postprandial nutrient influx.

Collectively, these findings support the concept of hepatic metabolic memory, whereby early hormonal cues elicit lasting molecular and functional adaptations that sensitize the liver to subsequent nutrient challenges. This memory likely involves coordinated transcriptional reprogramming, enzyme activity modulation, substrate availability shifts, and potentially epigenetic modifications (35). Understanding the cellular and molecular underpinnings of this memory will be critical to harnessing it therapeutically. Clinically, these results have important implications for individuals with type 2 diabetes or impaired early-phase insulin secretion, where inadequate morning insulin surges may impair hepatic priming and exacerbate postprandial hyperglycemia throughout the day (36). Therapeutic strategies aimed at restoring or mimicking hepatic insulin priming, whether via hepato-preferential insulin delivery, incretin-based agents, or meal timing optimization, could enhance hepatic glucose handling. Furthermore, insulin priming may interact with hepatic circadian rhythms, as the liver's metabolic programs are regulated by an endogenous clock anticipating feeding-fasting cycles (37). Disruption of this synchrony, common in shift work or metabolic syndrome, may blunt the priming effect and

worsen glucose intolerance (38-41). Future research should explore the crosstalk between insulin signaling and circadian regulators to develop interventions that restore metabolic homeostasis.

In conclusion, a physiological rise in morning insulin initiates a coordinated hepatic program that enhances glucose uptake, utilization, and storage later in the day. This priming effect engages both insulin-dependent and insulin-independent mechanisms, including hepatic glucose effectiveness and the portal glucose signal. Collectively, the data demonstrate that morning hyperinsulinemia equally enhances afternoon insulin and glucose action, suggesting that insulin priming likely targets shared cellular pathways. These findings provide a mechanistic basis for the second meal phenomenon and highlight the liver as a dynamic integrator of prior hormonal cues that shape its metabolic responsiveness. By revealing how early insulin exposure primes hepatic glucose metabolism, this work offers new opportunities to improve glycemic control in metabolic disease.

Acknowledgments

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.

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Table 1 – Mean hormone and substrate clamp data

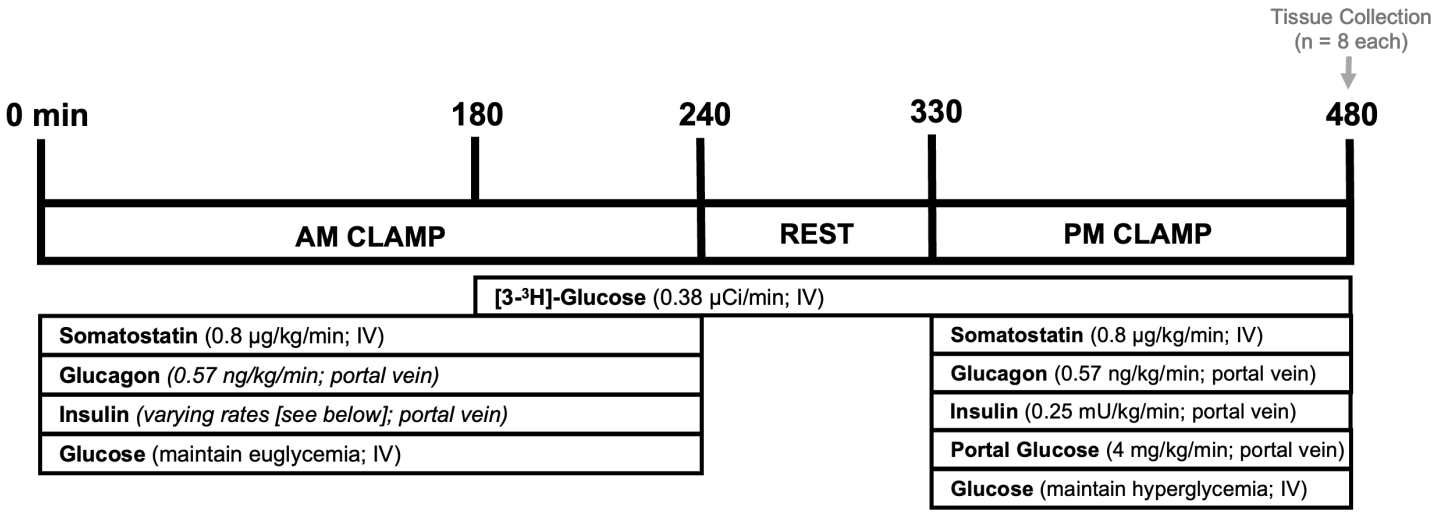
	Experimental Group	
AM Clamp Parameter (mean 30-240 min)	**No Prime (n=8)**	**Prime (n=8)**
Hepatic Sinusoidal Plasma Insulin (µU/mL)	18 ± 1	106 ± 6
Glucose Infusion Rate (mg/kg/min)	0.4 ± 0.3	8.8 ± 0.8
Arterial Plasma Glucagon (pg/mL)	22 ± 2	21 ± 2
Hepatic Sinusoidal Plasma Glucagon (pg/mL)	29 ± 2	29 ± 2
PM Clamp Parameter (mean 360-480 min)		
Arterial Plasma Insulin (µU/mL)	6 ± 1	6 ± 1
Hepatic Sinusoidal Plasma Insulin (µU/mL)	20 ± 1	24 ± 2
Arterial Plasma Glucagon (pg/mL)	21 ± 1	20 ± 1
Hepatic Sinusoidal Plasma Glucagon (pg/mL)	29 ± 1	27 ± 2

Figure Legends

1. **Figure 1: Experimental protocol.** Canines (*n*=8/group) underwent a 4h euglycemic clamp in the morning (0 to 240 min) with either basal insulin (No Prime) or hyperinsulinemia (Prime). Near the end of the AM clamp (180 min), tracer infusion began 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. The dogs then underwent a 2.5h PM hyperglycemic clamp with portal glucose delivery and basal insulin (330 to 480 min). Tissue collection occurred at the end of the PM clamp. IV; intravenous infusion.
2. **Figure 2: Morning (AM) clamp glucose and insulin flux data.** Arterial plasma glucose (A) and insulin (B), hepatic sinusoidal plasma insulin (D), and the amount of exogenous glucose required to maintain euglycemia (F) are shown for the No Prime and Prime groups; *n*=8/group. Bar graphs indicate the area under the curve (AUC) for arterial insulin concentrations (C), hepatic sinusoidal insulin concentrations (E), and glucose infusion rates (G) during the 4h AM period. Data are expressed as mean ± SEM. ****P<0.0001 between groups. ns = non-significant.
3. **Figure 3: Afternoon (PM) clamp glucose and insulin data.** A vertical line at 330 min separates the resting period from the onset of the PM clamp. Arterial plasma glucose (A), hepatic glucose load (B), the difference between the artery and the portal vein plasma glucose levels (C), and hepatic sinusoidal plasma insulin (D) are shown for the 2.5h euinsulinemic-hyperglycemic PM clamp period in the No Prime and Prime 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 euinsulinemic-hyperglycemic 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), net glycolytic/gluconeogenic flux (G), and net glycogenesis/glycogenolysis flux (H) are shown over time for the No Prime and Prime groups, *n*=8/group. The respective AUCs for the glucose infusion rate, non-hepatic glucose uptake, and net hepatic glucose uptake are shown for both groups (B, D, F). Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 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 output (H) are shown for the PM euinsulinemic-hyperglycemic clamp for the No Prime and Prime groups, *n*=8/group. Data are expressed as mean ± SEM. **P<0.01 between groups. ns = non-significant.
6. **Figure 6: Glycogen and terminal hepatic tissue gene expression data.** Tracer-determined direct glycogen synthesis during the PM clamp (A), terminal hepatic glycogen reserves (B), phosphoenolpyruvate carboxykinase 1 (PCK1) mRNA (C), glucose-6-phosphatase catalytic subunit 1 (G6PC1) mRNA (D), and liver-specific glycogen phosphorylase (PYGL) mRNA (E), glucokinase (GCK) mRNA (F), sterol regulatory element-binding protein 1c (SREBP-1c) mRNA (G), and fatty acid synthase (FAS) mRNA (H) levels are shown for the No Prime and Prime groups, *n*=8/group, as well as basal tissue when applicable (*n*=5). Data are expressed as mean ± SEM. *P<0.5, **P<0.01, ***P<0.001, ****P<0.0001 between groups. ns = non-significant.

7. **Figure 7: Summary of PM net hepatic glucose uptake (NHGU) data. A)** Mean PM NHGU data from control experiments previously completed. One of these groups (grey bar) received basal insulin in the AM with a PM HIHG clamp. The other (blue bar) received hyperinsulinemia in the AM with the same PM HIHG clamp. The size of the priming effect of AM insulin on PM NHGU is the difference in PM NHGU between these two groups, which is 3.8 mg/kg/min (shown in red). **B)** Mean PM NHGU data from the current experiments. The No Prime group (orange) received basal insulin in the AM with a PM hyperglycemic clamp with basal insulin delivery. The Prime group (green) received AM hyperinsulinemia with the same euinsulinemic-hyperglycemic clamp in the PM. The only difference between these two groups and the control groups presented in (A) is that they underwent a PM hyperglycemic clamp with **basal** insulin rather than having an elevation in insulin. The impact of the AM insulin prime on the PM effects of glucose (glucose effectiveness (GE) and the portal glucose signal (PGS) is equal to the difference in PM NHGU between the No Prime and Prime groups, which is equal to 2.1 mg/kg/min (shown in purple). **C)** Of the 3.8 mg/kg/min enhancement in PM NHGU following an AM insulin prime, 45% of that (1.7 mg/kg/min) was due to an enhancement in PM insulin action, whereas the other 55% (2.1 mg/kg/min) was due to the enhancement in PM glucose action.

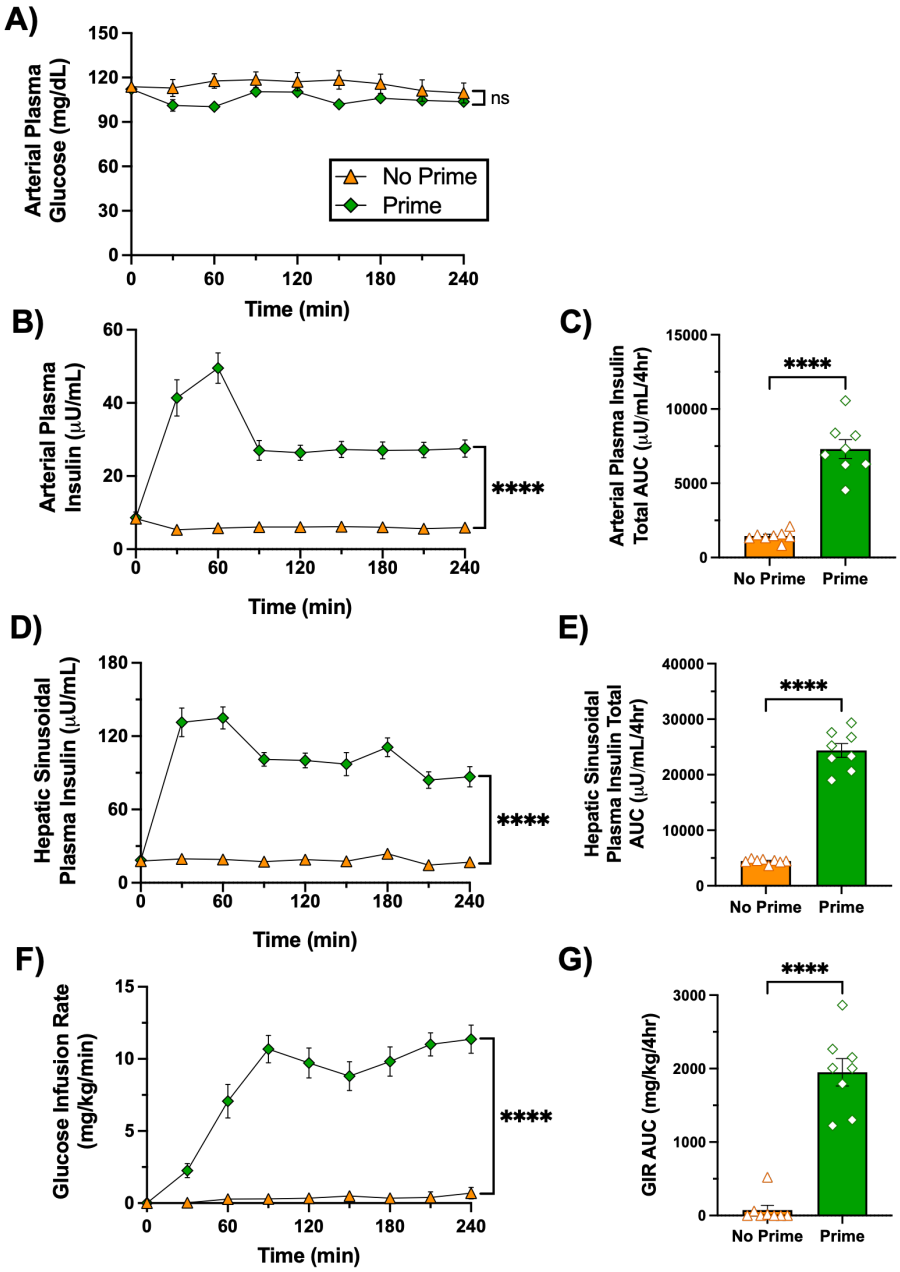


AM CLAMP INSULIN RATES (mU/kg/min):

No Prime (Basal insulin, *n=8*):
0-240 min: 0.25

Prime (Hyperinsulinemia, *n=8*):
0-30 min: 2.1
30-60 min: 2.4
60-240 min: 1.5

Figure 1: Experimental protocol. Canines (*n*=8/group) underwent a 4h euglycemic clamp in the morning (0 to 240 min) with either basal insulin (No Prime) or hyperinsulinemia (Prime). Near the end of the AM clamp (180 min), tracer infusion began 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. The dogs then underwent a 2.5h PM hyperglycemic clamp with portal glucose delivery and **basal** insulin (330 to 480 min). Tissue collection occurred at the end of the PM clamp. IV; intravenous infusion.



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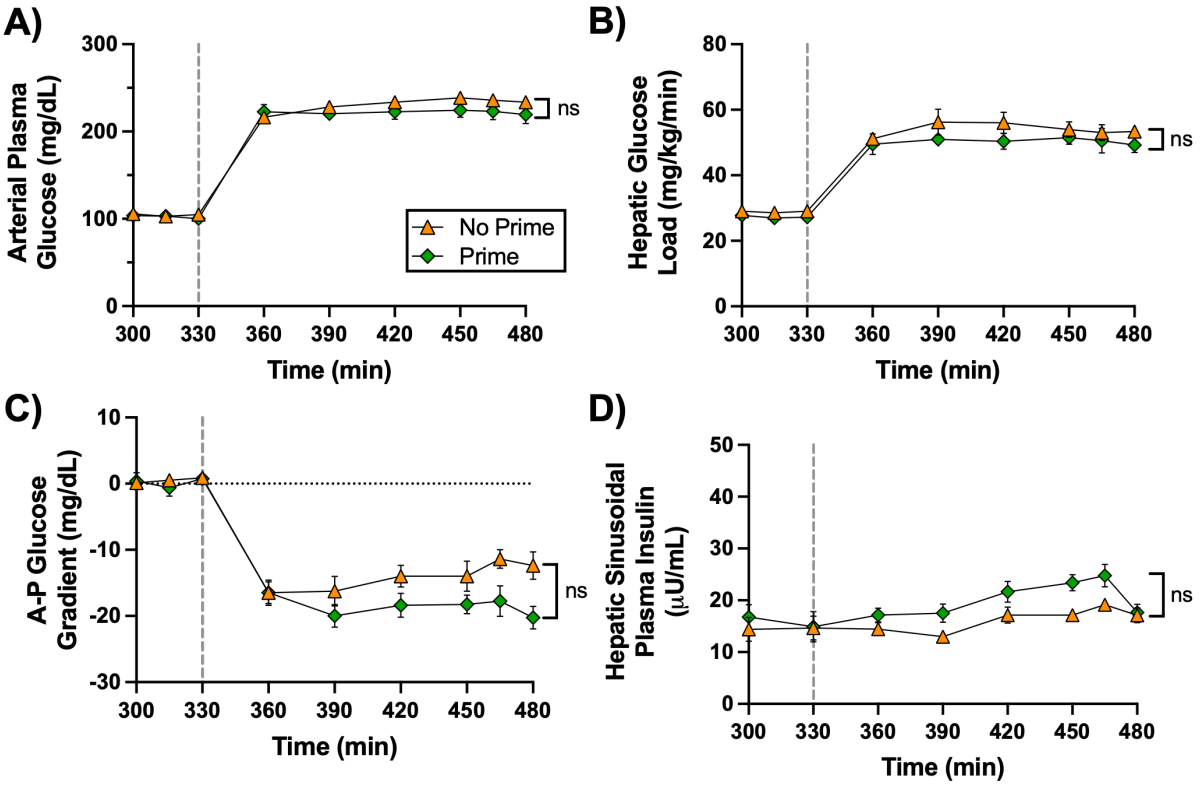

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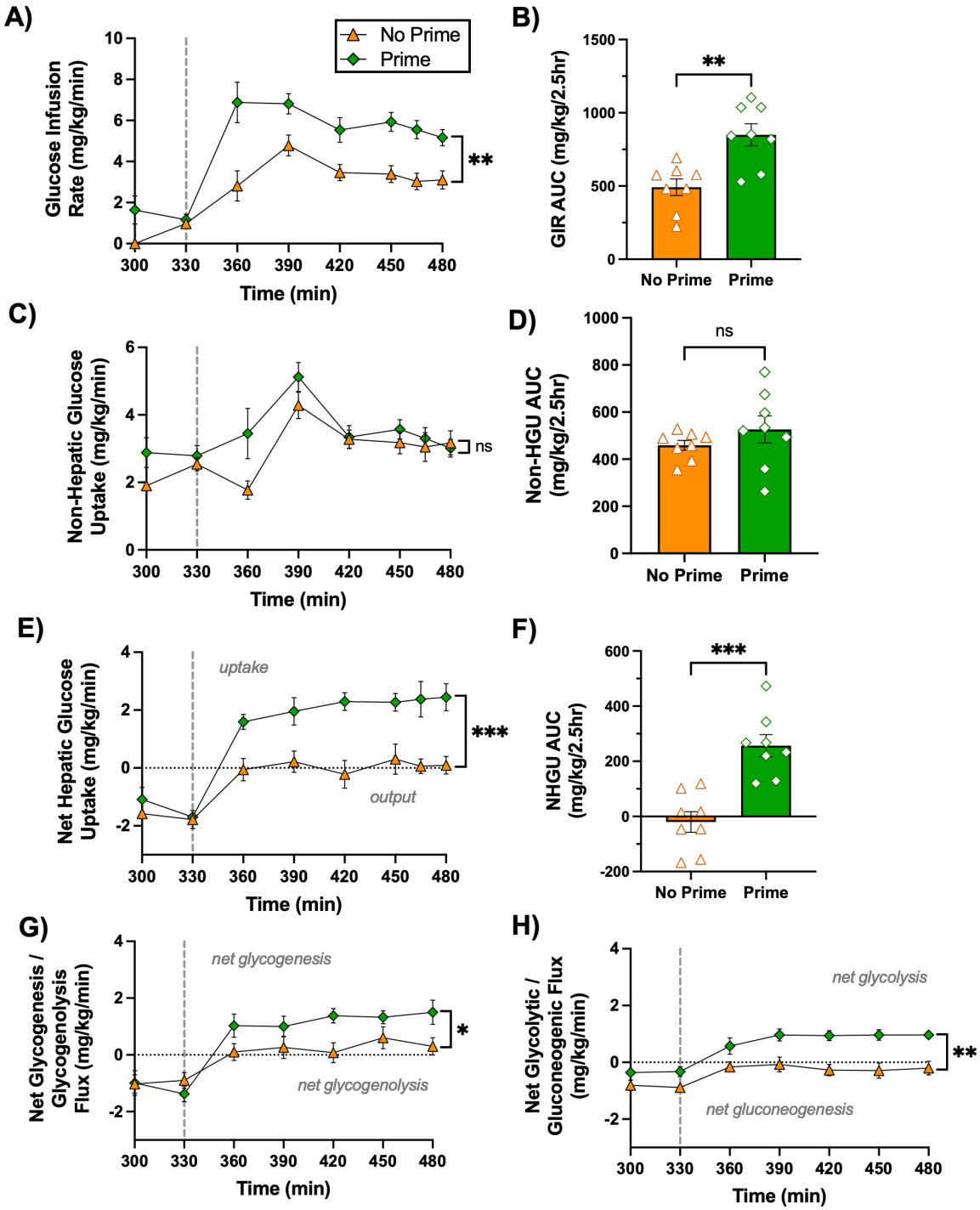

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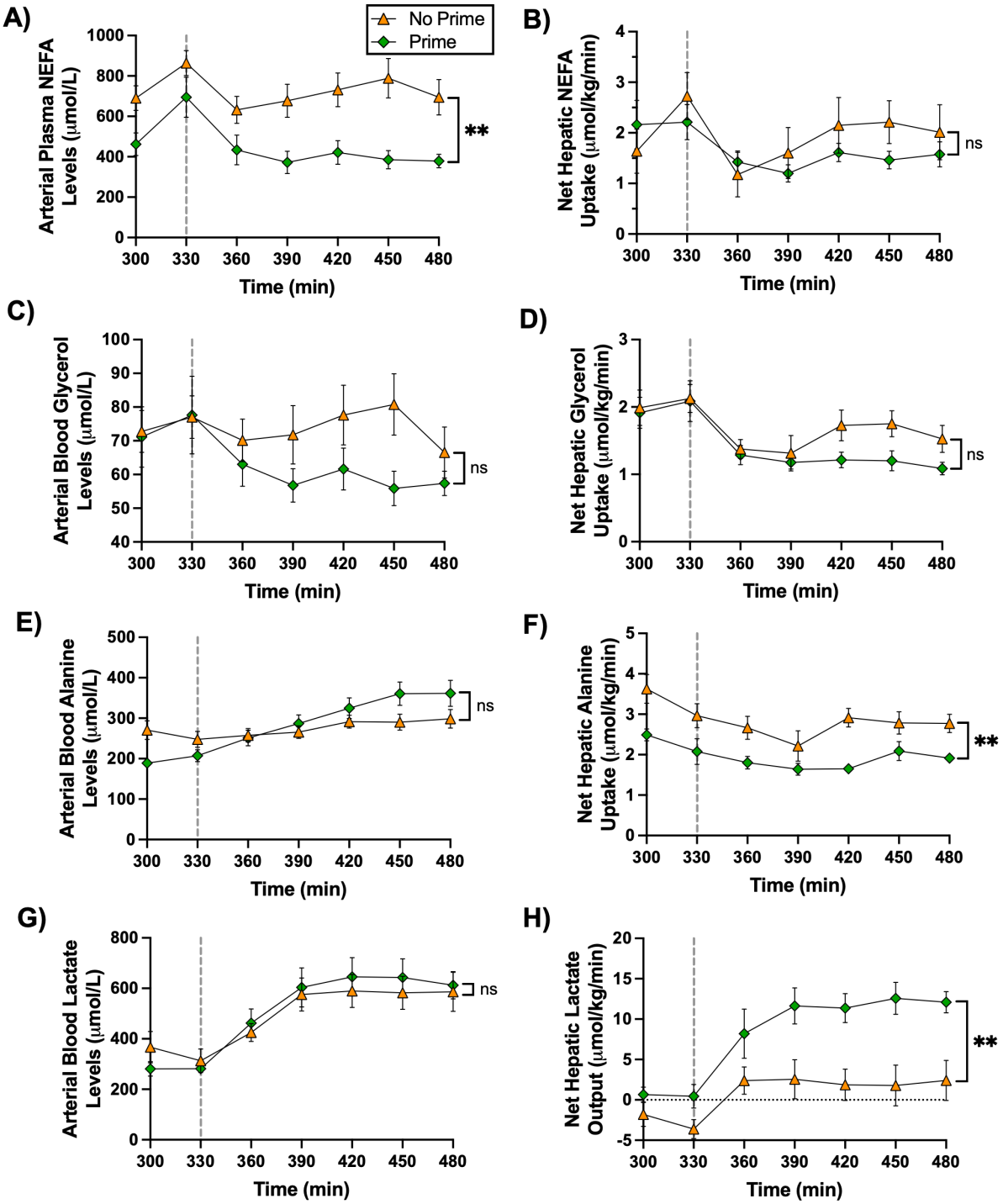

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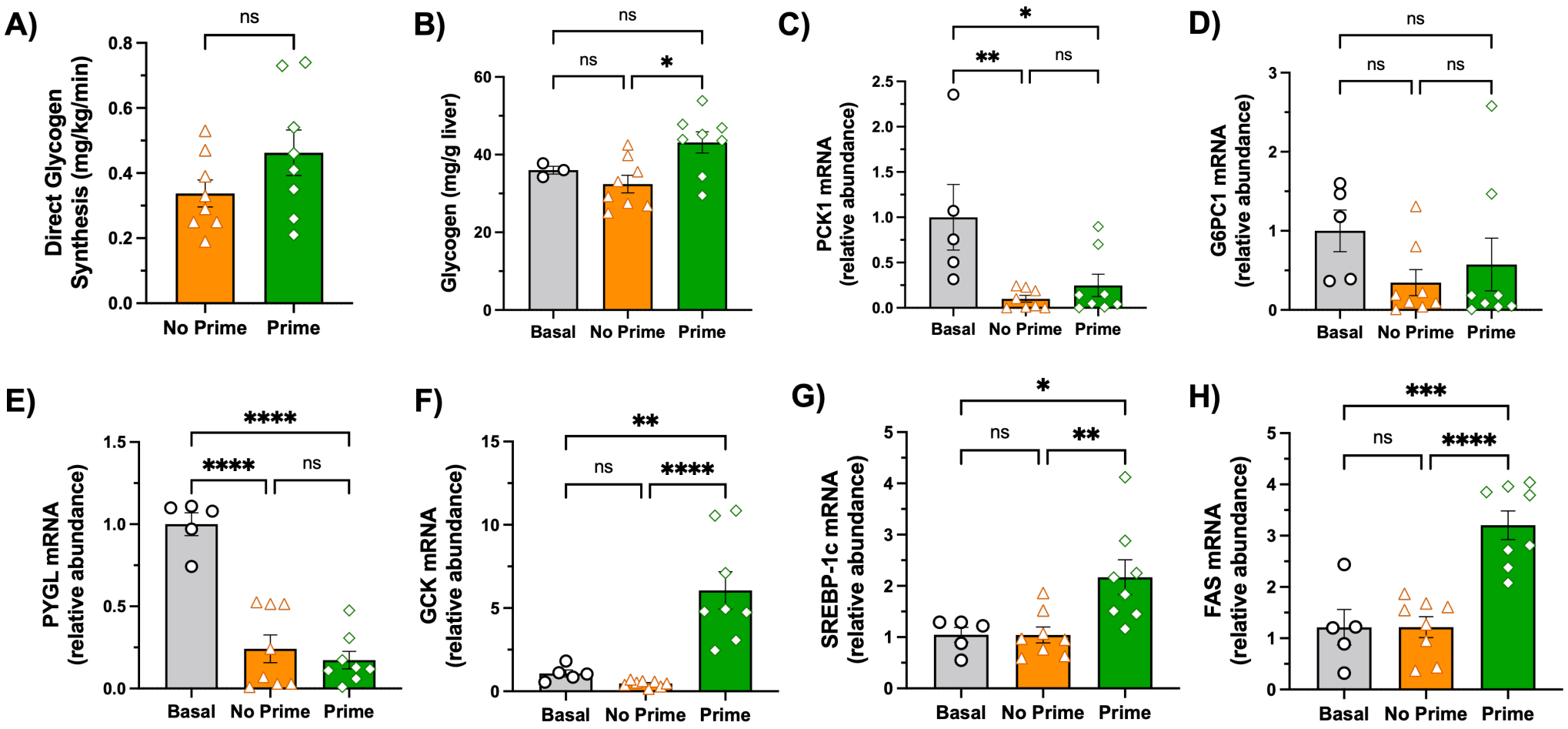

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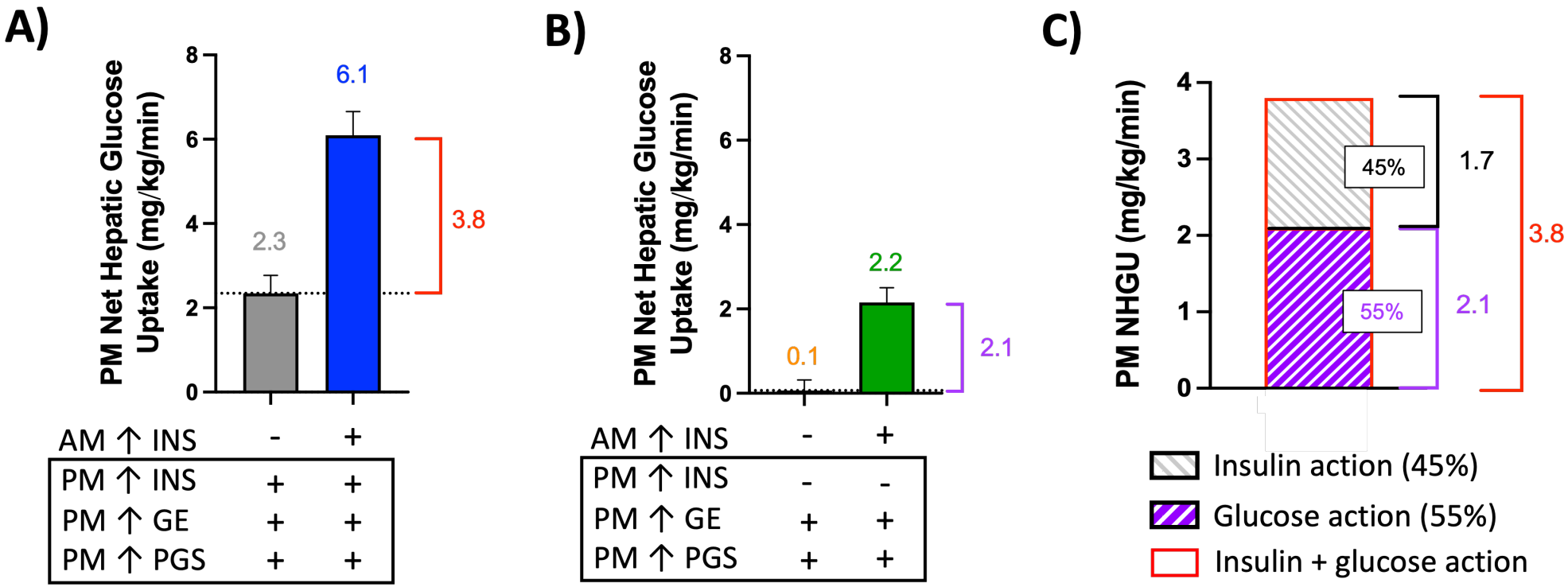

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