Morning Elevation in Insulin Enhances Afternoon Hepatic Postprandial Glucose Disposal in the Dog by Increasing Both Insulin Signaling and Glucose Action

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Abstract

The second-meal phenomenon refers to the improved glycemic response to a subsequent identical meal. Postprandial net hepatic glucose uptake (NHGU) is governed by the combined effects of three regulatory factors: insulin action (IA), initiated by hyperinsulinemia; glucose effectiveness (GE), driven by hyperglycemia; and the portal glucose signal (PGS), activated by glucose delivery into the hepatoportal circulation. Previous studies demonstrated that morning (AM) hyperinsulinemia primes the liver, causing substantially enhanced NHGU later in the day; however, it remained unclear which component of the afternoon (PM) response is augmented. To address this, we assessed how AM insulin elevation influences PM IA, GE, and the PGS. Dogs underwent an AM clamp with either a 4h hyperinsulinemic prime (Prime, $n=8$) or basal insulin delivery (No Prime, $n=8$). After a 1.5-hour rest, both groups underwent a PM hyperglycemic clamp (with portal glucose delivery) under basal insulin conditions. During the PM clamp, NHGU was significantly greater in the Prime versus No Prime group (2.2±0.3 vs. 0.1±0.3 mg/kg/min, $P<0.005$), indicating priming enhanced GE and/or PGS effects. In prior experiments with all three stimuli present in the PM (IA, GE, and PGS), AM insulin priming increased PM NHGU by 3.8 mg/kg/min. Thus, while AM insulin priming alone enhanced GE and/or PGS, the full effect requires an elevation in PM insulin, suggesting that morning insulin exposure primes the liver by augmenting both afternoon insulin action and glucose action.

Introduction

Effective postprandial glucose disposal is essential for maintaining metabolic health, and impairments in this process are a defining feature of insulin resistance and type 2 diabetes (1-3). An incompletely understood aspect of glucose metabolism is the second meal phenomenon, in which the glycemic response to a second, identical meal is attenuated compared with its initial response to the meal (4-6), suggesting that prior metabolic or hormonal cues influence later glucose handling. Consistent with this concept, we previously demonstrated in dogs that a rise in morning (AM) insulin enhanced net hepatic glucose uptake (NHGU) during an afternoon (PM) hyperinsulinemic-hyperglycemic clamp (7; 8). These findings indicate that early insulin exposure can prime the liver for more efficient glucose disposal later in the day, exerting effects that persist beyond the period of elevated circulating insulin.

Hepatic glucose uptake, storage, and release strongly influence postprandial glucose excursions, highlighting the liver's central role in glucose homeostasis (9). Notably, under physiologic conditions the liver can dispose of as much postprandial glucose as muscle (10). NHGU is regulated by the integrated actions of three primary signals: insulin action (IA), triggered by hyperinsulinemia; glucose effectiveness (GE), driven by hyperglycemia; and the portal glucose signal (PGS), activated by elevated glucose concentrations in the hepatoportal circulation, such as occurs with absorption of glucose from the gut (11; 12). Insulin promotes NHGU by suppressing endogenous glucose production and stimulating glycogen synthesis through well-characterized signaling pathways (13; 14). GE reflects the ability of glucose per se to suppress hepatic glucose output and enhance NHGU in the presence of basal insulin (15-17). The PGS arises from neural sensing of glucose delivery through the portal vein,

enabling the liver to anticipate nutrient influx and adjust glucose uptake and storage accordingly, via mechanisms that are less well defined (11; 18-20). Together, these factors enable the liver to function as an active integrator of postprandial glucose signals (12).

Despite evidence that prior insulin exposure enhances NHGU later in the day, it remains unclear which afternoon regulatory signals (IA, GE, and/or the PGS) are augmented by morning insulin exposure. This distinction is particularly relevant because both insulin action and glucose action are impaired in insulin-resistant and diabetic states, contributing to defective postprandial glucose disposal and exaggerated glycemic excursions (21; 22). Determining whether AM insulin exposure enhances the PM hepatic response to glucose, insulin, or their combined actions is therefore essential for defining how early hormonal cues regulate hepatic glucose metabolism and for identifying mechanisms that may be leveraged to improve postprandial glucose control in metabolic disease. To address this gap, we assessed how an elevation in morning insulin influences the effects of glucose (GE and the PGS) vs. insulin (IA) during a PM euinsulinemic-hyperglycemic clamp.

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 inferior vena cava and the splenic and jejunal veins, which supply the hepatic portal vein. These catheters were secured subcutaneously until the day of the experiment, when they were exteriorized under local anesthesia. 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 min, followed by a 90 min break before the PM clamp, which took place from 330 to 480 min **(Fig. 1).** Blood samples were collected every 15 to 30 minutes from the femoral artery, hepatic

portal vein, and the left major 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 to maintain 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 (insulin was 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) (7). The other group, No Prime, received intraportal basal insulin replacement in the morning (0.25 mU/kg/min). Prior research from our lab has shown that the enhancement of HGU in the afternoon is driven by an increase in AM insulin levels, rather than a rise in morning glucose levels (7). Therefore, arterial plasma glucose was clamped at euglycemia (~110 mg/dL) for the entirety of the AM clamp. To allow for tracer-determined direct glycogen synthesis measurements in the afternoon, 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 in the PM with insulin and glucagon kept basal **(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 basal insulin (0.25 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 (11). To further investigate the liver's metabolic response, all dogs were anesthetized with infusions ongoing at the end of the PM clamp (480 min). Hepatic tissue was then rapidly excised, flash-frozen in liquid nitrogen, and stored at -80°C for subsequent molecular analyses. The animals were then euthanized.

Analyses

Biochemical and Molecular Methods

Whole blood samples were collected to assess hormone and substrate flux across the liver using arteriovenous balance techniques (23). Plasma glucose levels were measured in quadruplicate throughout the experiment using an Analox GM9 glucose analyzer. Plasma insulin (#PI-12K, MilliporeSigma, Burlington, MA), glucagon (#GL-32K, MilliporeSigma), and cortisol (VUMC Analytical Services in-house primary antibody with I125 cortisol from MP Biomedicals, Santa Ana, CA) were quantified by radioimmunoassay (23). Glucagon values were background corrected to account for non-specific binding, ensuring that measured concentrations accurately reflected circulating hormone levels.

Key metabolic intermediates, including lactate, glycerol, alanine, and non-esterified fatty acids (NEFA), were measured using enzymatic spectrophotometric assays (23). The specific activity of [3-^{3}H]-glucose in plasma samples was determined following deproteinization using the Somogyi method, with quantification by liquid scintillation counting (24). Terminal hepatic glycogen content was measured using the Keppler and Decker amyloglucosidase method (25).

At the end of the PM clamp, hepatic tissue was collected for molecular analyses, including qPCR, Western blotting, and enzymatic activity assays. A group of overnight-fasted dogs were used as basal controls, as applicable ($n=5$). Gene expression was normalized to GAPDH, and protein levels were standardized to total protein using Ponceau S staining (23). All assays were optimized for canine-specific targets using validated primers and antibodies (23). Glucokinase activity was measured using an

assay adapted from Shiota et al. (26). Detailed protocols, including reagents, primers, antibodies, sample preparation, and calculations, are provided in the supplemental materials.

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. Measurements obtained in plasma were converted to whole blood values using a conversion factor of 0.75, as previously described (23). Negative values of NHGB indicate net glucose uptake, meaning that hepatic glucose uptake exceeds glucose production. This approach was also used 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 correcting 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 during the PM clamp. This was measured

in disintegrations per minute (dpm) of labeled glucose incorporated into glycogen from 330-480 min. The specific activity of the glucose precursor pool was determined by the ratio of labeled to unlabeled glucose (dpm/mg) in the inflowing blood, weighted by 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 (27; 28). Previous studies have shown that hepatic glucose oxidation is minimal, approximately 0.2 mg/kg/min under the hyperinsulinemic-hyperglycemic clamp conditions seen in these dogs, contributing little to overall hepatic glucose flux (29; 30). Glycolytic flux was estimated by adding HGO to net hepatic lactate output. 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 to hepatic blood flow, following established methodology.

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, and molecular outcomes were analyzed

using a one-way ANOVA with Tukey's post hoc correction. Normality of all datasets was confirmed using the Shapiro-Wilk test, and all data were found to follow a normal distribution. 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

During the AM clamp, plasma glucose was maintained at euglycemia (105-115 mg/dL) in both groups **(Fig. 2A).** Arterial plasma insulin was maintained at basal levels in the No Prime group (6 ± 1 µU/mL), whereas insulin was elevated in the Prime group according to the experimental design (32 ± 3 µU/mL; **Fig. 2B, C**). As a result, hepatic sinusoidal insulin concentrations were approximately sixfold higher in the Prime group than in the No Prime group (**Fig. 2D, E; Table 1**). Arterial and hepatic sinusoidal glucagon concentrations remained basal and did not differ between groups **(Table 1).** Consistent with the higher insulin exposure, the Prime group required significantly more exogenous glucose to maintain euglycemia during the AM clamp than the No Prime group **(Fig. 2F, G; Table 1).** Thus, the AM clamp successfully established sustained hyperinsulinemia in the Prime group without altering glucose or glucagon levels.

PM Clamp Glucose, Hormone, and Metabolite Data

During the PM clamp, experimental conditions were identical between groups. Arterial plasma glucose was clamped at hyperglycemia (230-240 mg/dL; **Fig. 3A, B**), the portal glucose signal was activated by intraportal glucose delivery resulting in a

negative arterial-to-portal glucose gradient **(Fig. 3C),** and arterial and hepatic sinusoidal insulin concentrations were maintained at basal levels **(Fig. 3D; Table 1).** Despite matched PM clamp conditions, the Prime group required 52% more glucose to maintain hyperglycemia than the No Prime group (glucose infusion rate of 7.0 ± 0.4 vs. 4.6 ± 0.4 mg/kg/min, respectively; **Fig. 4A, B).** This difference was not attributable to changes in non-hepatic glucose uptake, which did not differ between groups **(Fig. 4C, D).** Instead, the increased glucose requirement was due to a marked increase in net hepatic glucose uptake (NHGU) in the Prime group (2.2 ± 0.3 vs. 0.1 ± 0.3 mg/kg/min in Prime vs. No Prime; **Fig. 4E, F).** The enhancement in NHGU in the Prime group reflected both increased net glycolytic flux **(Fig. 4G)** and increased net glycogenesis **(Fig. 4H).** Thus, morning insulin priming resulted in a sustained amplification of PM hepatic glucose uptake even if insulin did not rise during the PM clamp.

Arterial non-esterified fatty acid (NEFA) concentrations were significantly higher in the No Prime group than in the Prime group during the PM clamp, whereas net hepatic NEFA uptake did not differ between groups **(Fig. 5A, B).** Arterial glycerol concentrations and net hepatic glycerol uptake were similar between groups **(Fig. 5C, D).** Arterial alanine concentrations were also comparable; however, net hepatic alanine uptake was reduced modestly in the Prime group relative to the No Prime group **(Fig. 5E, F).** Arterial lactate concentrations did not differ between groups, but net hepatic lactate output was approximately fivefold greater in the Prime group than in the No Prime group **(Fig. 5G, H),** consistent with increased hepatic glucose uptake and glycolytic flux.

Glycogen Content and Hepatic Gene and Protein Analyses

Terminal hepatic glycogen content was significantly greater in the Prime group compared with the No Prime group (43 ± 3 vs. 36 ± 1 mg glycogen/g liver; **Fig. 6A**). Direct glycogen synthesis during the PM clamp, assessed by incorporation of tritiated glucose into hepatic glycogen, tended to be greater in the Prime group but this change did not reach statistical significance **(Fig. 6B).** Phosphorylated Akt relative to total Akt was similar in both the Prime and No Prime groups compared with basal controls, consistent with basal insulin conditions during the PM clamp **(Fig. 6C).** *Glucokinase* (*GCK*) mRNA abundance was significantly greater in the Prime group than in the No Prime and basal groups at the end of the PM clamp **(Fig. 6D);** however, GCK protein abundance and enzymatic activity did not differ between groups **(Fig. 6E, F).** Glycogen synthase protein was similarly dephosphorylated and activated in both experimental groups relative to basal **(Fig. 6G),** while phosphorylated glycogen phosphorylase protein did not differ among groups **(Fig. 6H).** Transcript levels of *phosphoenolpyruvate carboxykinase 1* (*PCK1*) and *glucose-6-phosphatase catalytic subunit 1* (*G6PC1*) were similarly reduced in both Prime and No Prime groups compared with basal **(Supplemental Fig. 1A, 1B).** In contrast, *sterol regulatory element-binding protein-1c (SREBP-1c)* and *fatty acid synthase (FAS)* mRNA levels were significantly elevated in the Prime group relative to the No Prime group at the end of the PM clamp **(Supplemental Fig. 1C, 1D).**

Discussion

The second meal phenomenon, in which the glycemic response to a subsequent identical meal is improved, reflects the body's capacity to integrate prior hormonal and nutrient exposures to optimize later postprandial glucose handling (6; 31; 32). This adaptation provides a potential therapeutic avenue which is relevant for disease states where postprandial glucose disposal is reduced due to impaired insulin action and/or glucose effectiveness, such as type 1 and 2 diabetes (4; 6; 32-35). Previous studies in dogs demonstrated that it is the physiological rise in morning insulin which is the component of the first meal that primes the liver for enhanced NHGU and glycogen storage later in the day and that this effect requires activation of hepatic insulin receptors during the morning period (7; 8). Building on this foundation, the present study was designed to define which components of the afternoon hepatic response are enhanced by morning insulin exposure. This was done by isolating glucose-mediated regulation of NHGU (GE and the PGS), under basal insulin conditions in the PM, thereby distinguishing these effects from those of insulin action (11).

To address this question, we used a reductive experimental approach to identify which regulatory signals are enhanced by a morning insulin prime in controlling afternoon NHGU. Specifically, we compared the effect of morning insulin priming on afternoon hepatic glucose metabolism during a hyperinsulinemic-hyperglycemic clamp (36), either when all three regulatory factors were engaged, or when just GE and the PGS were elevated under basal insulin conditions. When afternoon IA, GE, and the PGS were all engaged, morning insulin priming increased afternoon NHGU by 3.8 mg/kg/min **(Fig. 7A).** In the present study, with matching hyperglycemia and portal glucose delivery in the two groups, but in the presence of basal insulin, morning priming

increased NHGU by 2.1 mg/kg/min **(Fig. 7B).** This pattern suggests that a substantial portion of the priming effect (about half) is mediated through enhanced responsiveness to glucose-mediated signals, whereas the remaining component likely reflects augmented afternoon insulin action (1.7 mg/kg/min) **(Fig. 7C).** However, this calculation relies on cross-study comparisons and assumes additivity based on historical experiments (37; 38). In reality, these pathways likely interact and may coordinate synergistically within integrated hepatic metabolic networks. The key point is not the precise quantitative partitioning of afternoon NHGU, but that morning insulin exposure amplifies the liver's sensitivity to both hormonal and glucose-mediated cues later in the day, enhancing postprandial glucose disposal. Thus, this study demonstrates that morning insulin augments both the glucose-mediated (GE/the PGS) and insulin-mediated components of afternoon NHGU.

Afternoon clamp conditions were carefully matched to control for confounding influences. Plasma glucose, portal glucose delivery, and basal insulin and glucagon replacement were tightly controlled in both groups with no statistical differences. Minor differences included a slightly greater relative arterial-portal glucose gradient in the Prime group (4 mg/dL) and a minimally higher hepatic sinusoidal insulin concentration in the Prime group (4 µU/mL). These differences were small in magnitude, however, and are unlikely to have meaningfully alter hepatic glucose metabolism.

Interestingly, primed animals showed significantly higher hepatic expression of the lipogenic transcription factor *SREBP-1c*, a well-established insulin-responsive transcription factor (39), and its downstream target, *FAS*, at the end of the PM

hyperglycemic clamp. Notably, this occurred under conditions in which the morning clamp produced a transient rise in insulin, followed later by hyperglycemia during the PM clamp, rather than the simultaneous high insulin and high glucose previously reported as necessary for hepatic activation (39; 40). Since gene expression was assessed only at the terminal time point, we cannot exclude the possibility that insulin exposure during the morning clamp initiated or partially activated these transcriptional programs. However, the persistence or amplification of *SREBP-1c* and *FAS* expression into the afternoon hyperglycemic period suggests that prior insulin exposure establishes a hepatic state that alters the transcriptional response to subsequent glucose availability. These findings point to a potentially novel mechanism in which temporal separation of hormonal and nutrient signals can coordinate carbohydrate and lipid metabolism. The coordinated activation of *SREBP-1c* and *FAS*, along with enhanced glycolytic flux and hepatic glycogen storage, reflects a hepatic memory of earlier insulin exposure and a shift toward integrated carbohydrate and lipid metabolism. This primed state provides a mechanistic context for the enhanced NHGU observed in primed animals, linking early insulin exposure to subsequent regulation of both glucose and lipid pathways. Despite this, circulating lipid availability did not parallel hepatic transcriptional responses. Although arterial NEFA concentrations were higher in the No Prime group, net hepatic NEFA uptake did not differ between groups, and afternoon lipolysis was not suppressed in either condition.

The enhancement of NHGU within the first 30 minutes of the PM clamp further indicates that the effects of morning insulin were already present at the time of glucose exposure rather than developing progressively during the clamp. Molecular

measurements obtained at the terminal time point therefore provide mechanistic context but cannot fully account for the dynamic flux changes observed during the clamp itself. At the end of the PM clamp, primed animals exhibited elevated hepatic *GCK* mRNA, whereas total GCK protein abundance, maximal GCK enzymatic activity, and the phosphorylation states of glycogen synthase and glycogen phosphorylase were similar between groups. The increase in *GCK* mRNA is consistent with insulin-dependent transcription initiated in the morning and relatively slow mRNA turnover during the afternoon hyperglycemic clamp and supports the concept that morning insulin establishes a molecular program that enhances glucose phosphorylation capacity during subsequent hyperglycemia (41-43).

Prior work from our group demonstrated that a physiological rise in morning insulin results in increased hepatic *GCK* mRNA and protein, elevations that persist through the intervening rest period (330 min) following the AM clamp (8). In the present study, the absence of elevated GCK protein at the terminal time point (480 min), despite a clear enhancement of NHGU in the Prime group (evident from 360-480 min), was unexpected. However, this discrepancy does not preclude a central role for glucokinase in mediating the priming effect. *In vivo*, GCK activity is regulated not only by total enzyme abundance but also by dynamic processes such as subcellular compartmentalization and protein-protein interactions. These include binding to glucokinase regulatory protein in the nucleus (44), as well as the bifunctional enzyme PFK-2/FBPase-2 (45) and the pro-apoptotic mitochondrial protein BAD (46). Through these interactions and others, GCK can be sequestered or released in response to glucose availability, thereby modulating its cytosolic activity depending on the specific

physiological context (47). Importantly, these complex regulatory mechanisms are not captured by terminal protein measurements or *ex vivo* activity assays. It is plausible that prior insulin exposure may sensitize these processes, although this remains to be demonstrated and represents an important avenue for future investigation. Furthermore, the rapid onset and sustained enhancement of NHGU argue against accelerated degradation or loss of functional GCK during the afternoon, particularly given its long hepatic half-life (48). Glucokinase remains the most plausible molecular mediator of morning insulin priming, as it catalyzes the rate-limiting step of hepatic glucose uptake under hyperglycemic conditions and uniquely integrates insulin-dependent transcription, a long protein half-life, and rapid activation by glucose (42; 49-51). Other hepatic proteins are less likely contributors, as they are not simultaneously insulin-inducible, long-lived, and acutely responsive to glucose availability later in the day (50).

In summary, these studies demonstrate that a physiological rise in morning insulin primes the liver to respond more effectively to a subsequent nutrient load, even without a rise in afternoon insulin. This finding is notable because it suggests that hepatic priming may confer metabolic benefit even in settings of limited or impaired insulin availability, such as in individuals with type 1 diabetes or insulin resistance / type 2 diabetes. Whether this priming mechanism is preserved or altered in metabolically dysfunctional states, however, remains to be determined. Under these conditions, the priming enhances glucose-mediated regulatory mechanisms, but full second-meal enhancement of NHGU, still requires a concurrent rise in afternoon insulin, indicating that morning insulin also potentiates later insulin action. Glucokinase is the most plausible molecular mediator, consistent with its insulin-dependent regulation and

central role in hepatic glucose phosphorylation. Together, these findings define a hepatic mechanism by which early-day insulin exposure shapes later postprandial glucose handling and suggest that strategically timed insulin or insulinotropic interventions could improve postprandial glycemic control in insulin-resistant and diabetic states by restoring defects in glucose effectiveness and insulin action.

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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.

Data availability. Values for all data points in graphs are reported in the Supporting Data Values file. Additionally, these data and supplemental materials are publicly available: doi.org/10.6084/m9.figshare.31049578. All other data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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

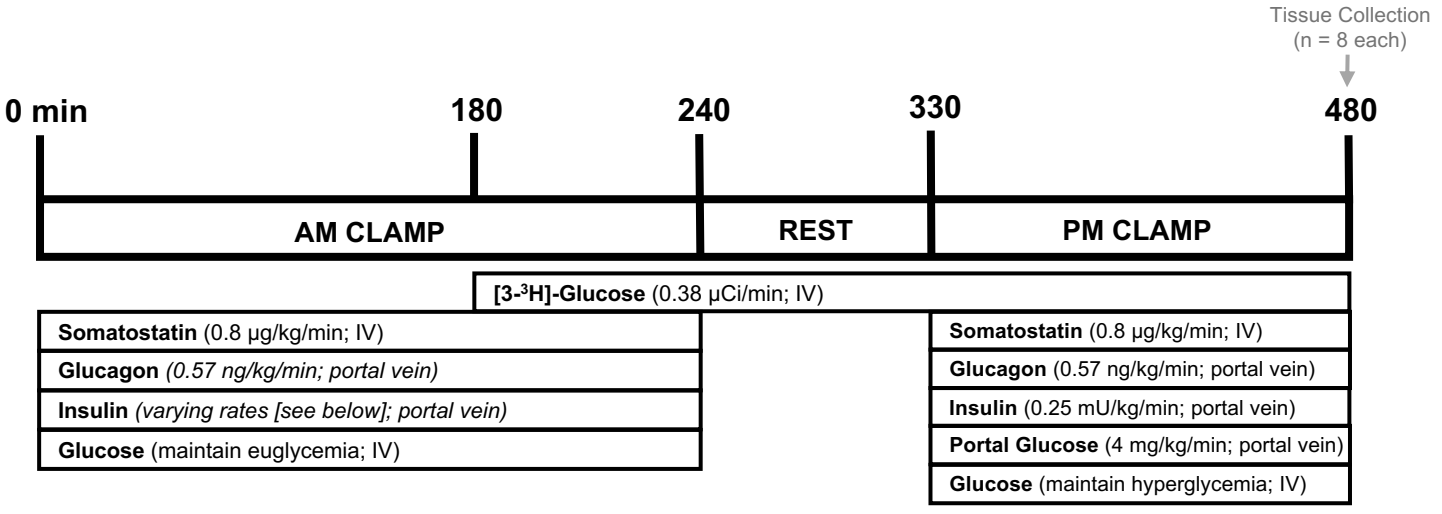
1. **Figure 1: Experimental protocol.** Canines (*n*=8/group) underwent a 4h euglycemic clamp in the morning (0 to 240 min) with either basal insulin (No Prime) or hyperinsulinemia (Prime). Near the end of the AM clamp (180 min), tracer infusion began to allow enough time for equilibration before the 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 delta 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 content and hepatic gene/protein expression at the end of the PM clamp.** Terminal hepatic glycogen content is shown in (A), and tracer-determined direct glycogen synthesis during the PM clamp is shown in (B). Protein and gene expression are shown as follows: Akt protein (phospho/total) in (C), glucokinase (GCK) mRNA in (D), GCK protein (normalized to Ponceau S stain) in (E), glucokinase (GCK) activity at 0.5 Vmax in (F; n = 5 or 6/group), glycogen synthase (GS) protein (phospho/total) in (G), and glycogen phosphorylase (GP) protein (phospho/total) in (H). Protein data were obtained by western blot and normalized to Ponceau S stain; representative images are provided in Supplemental Figure 1. Data are presented for the No Prime and Prime groups (n = 8/group), with basal tissue values included where applicable (n = 5). All values are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 between groups; ns = not significant.
7. **Figure 7: Summary of PM net hepatic glucose uptake (NHGU) data.** Mean PM NHGU data from control experiments previously completed are shown in (A). 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). Mean PM NHGU data from the current experiments are shown in (B). 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). 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 (C).

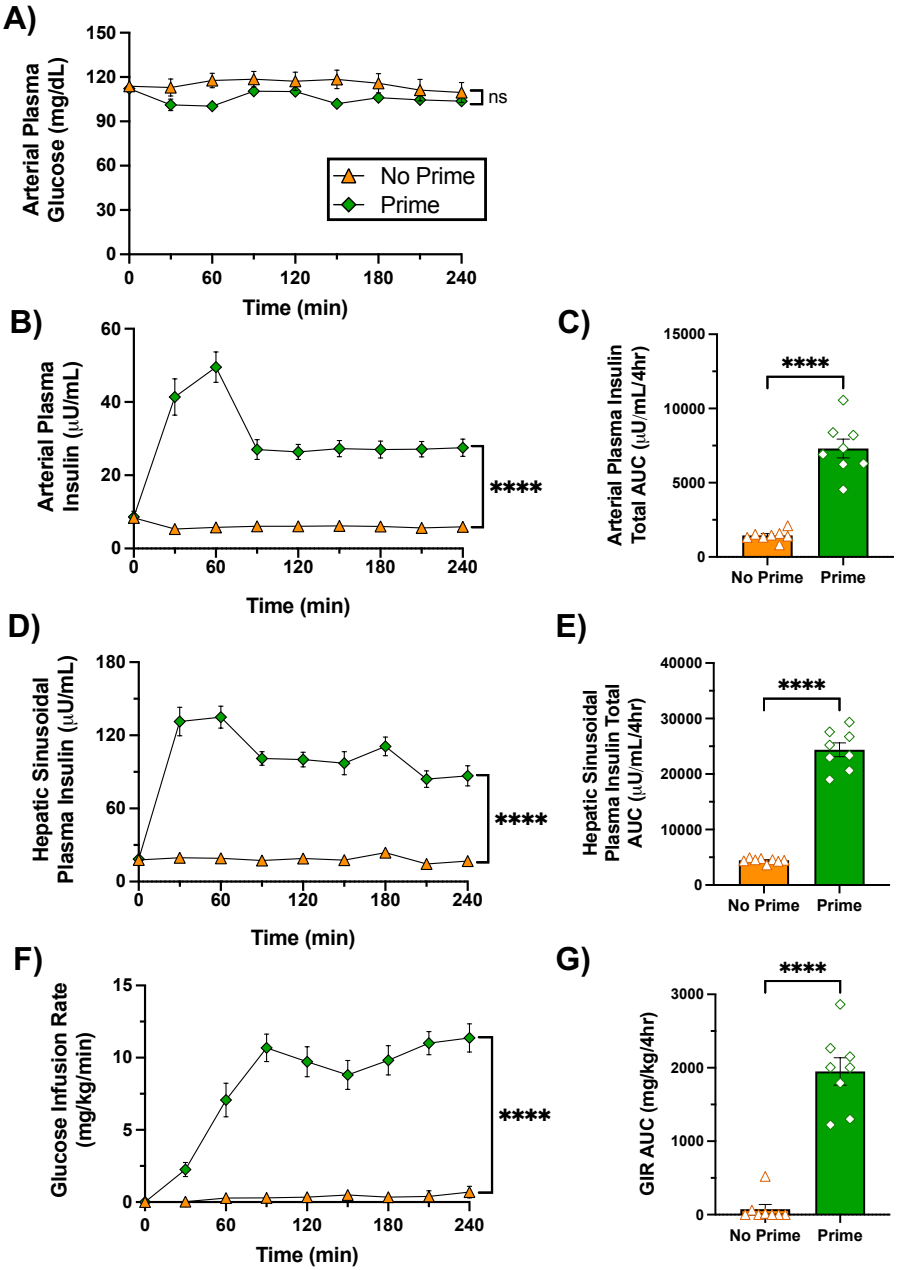


AM CLAMP INSULIN RATES (mU/kg/min):

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

Prime (Elevated insulin, *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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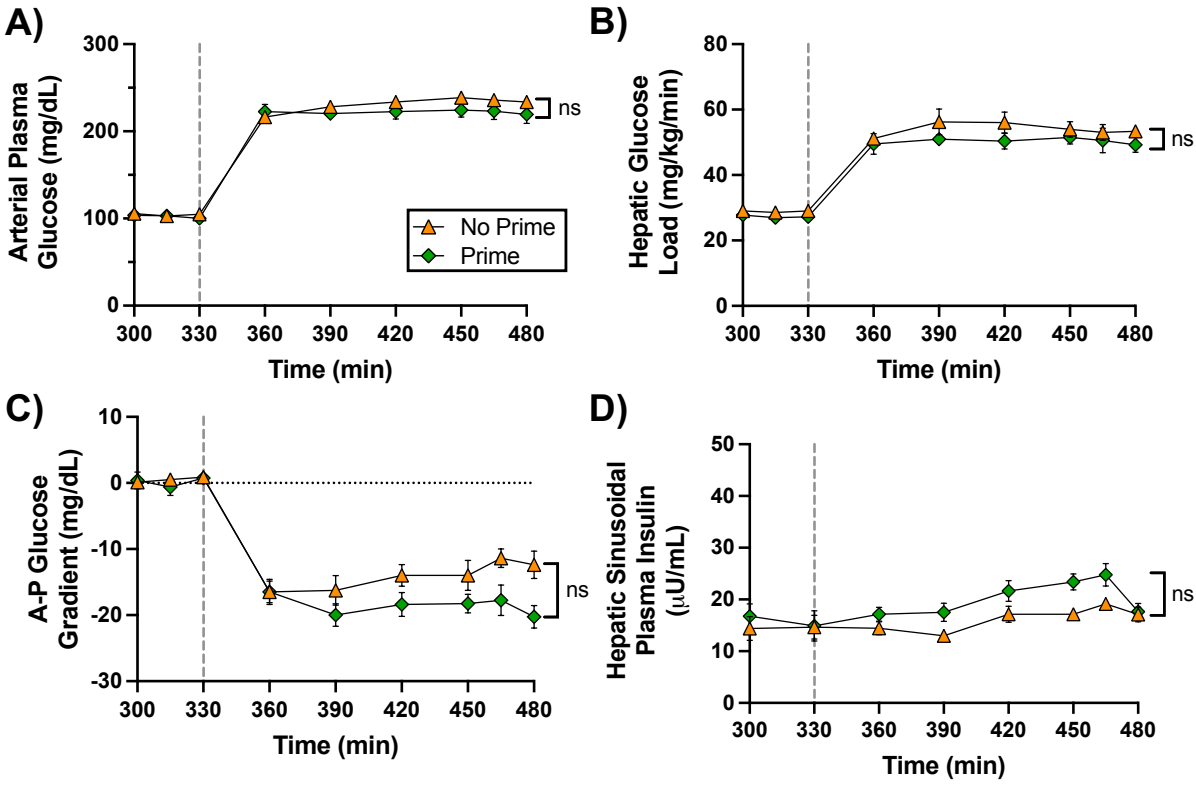

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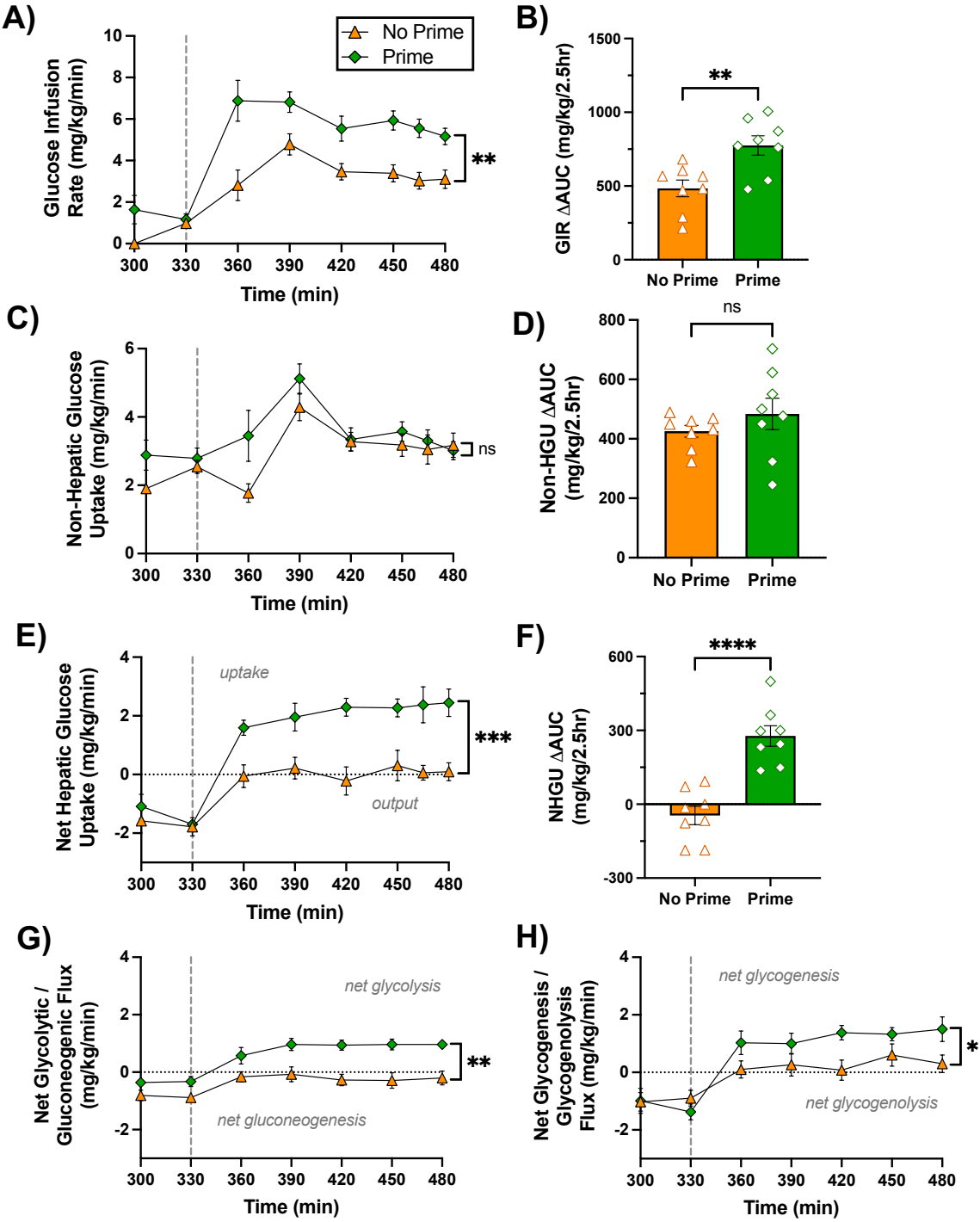

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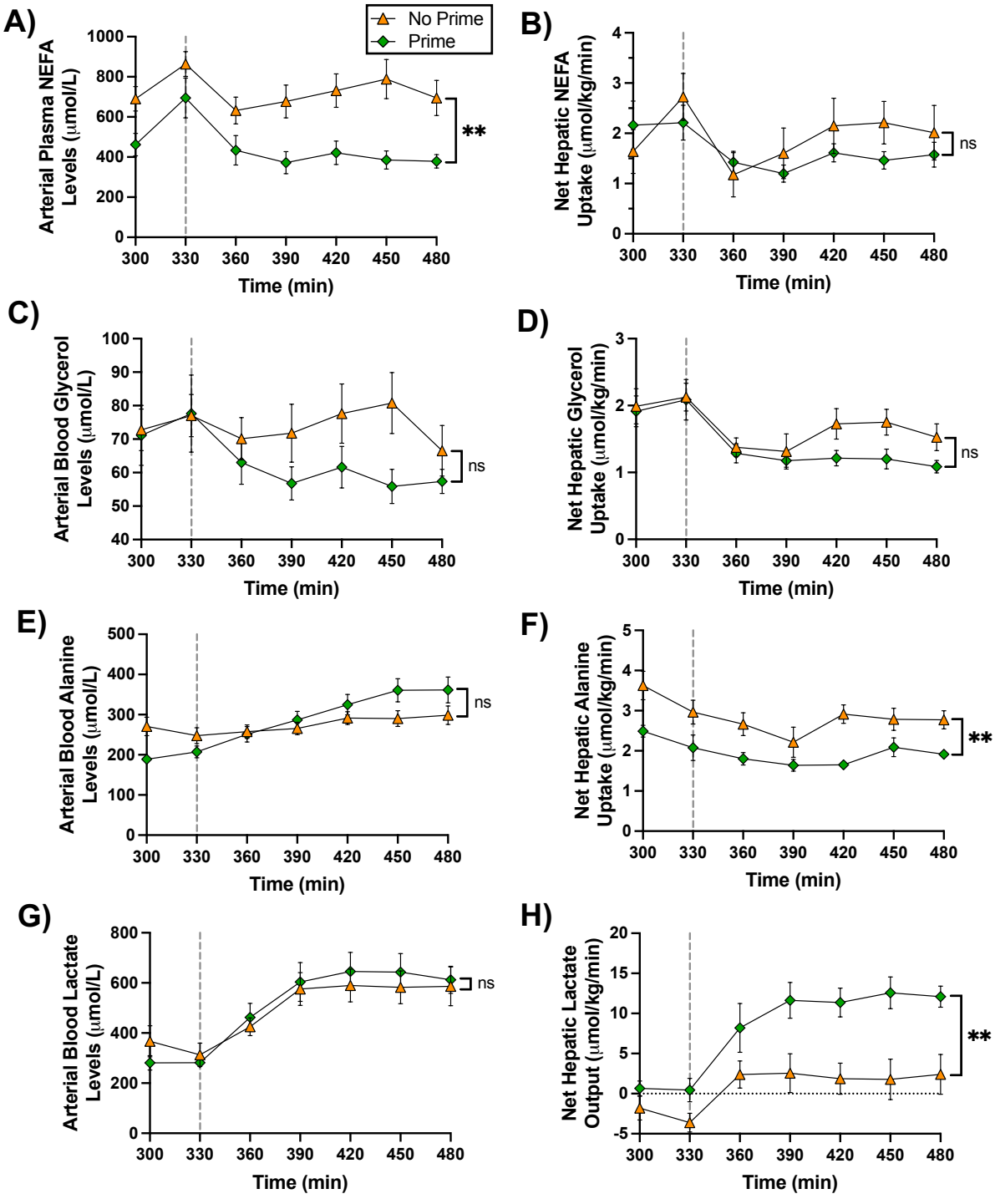

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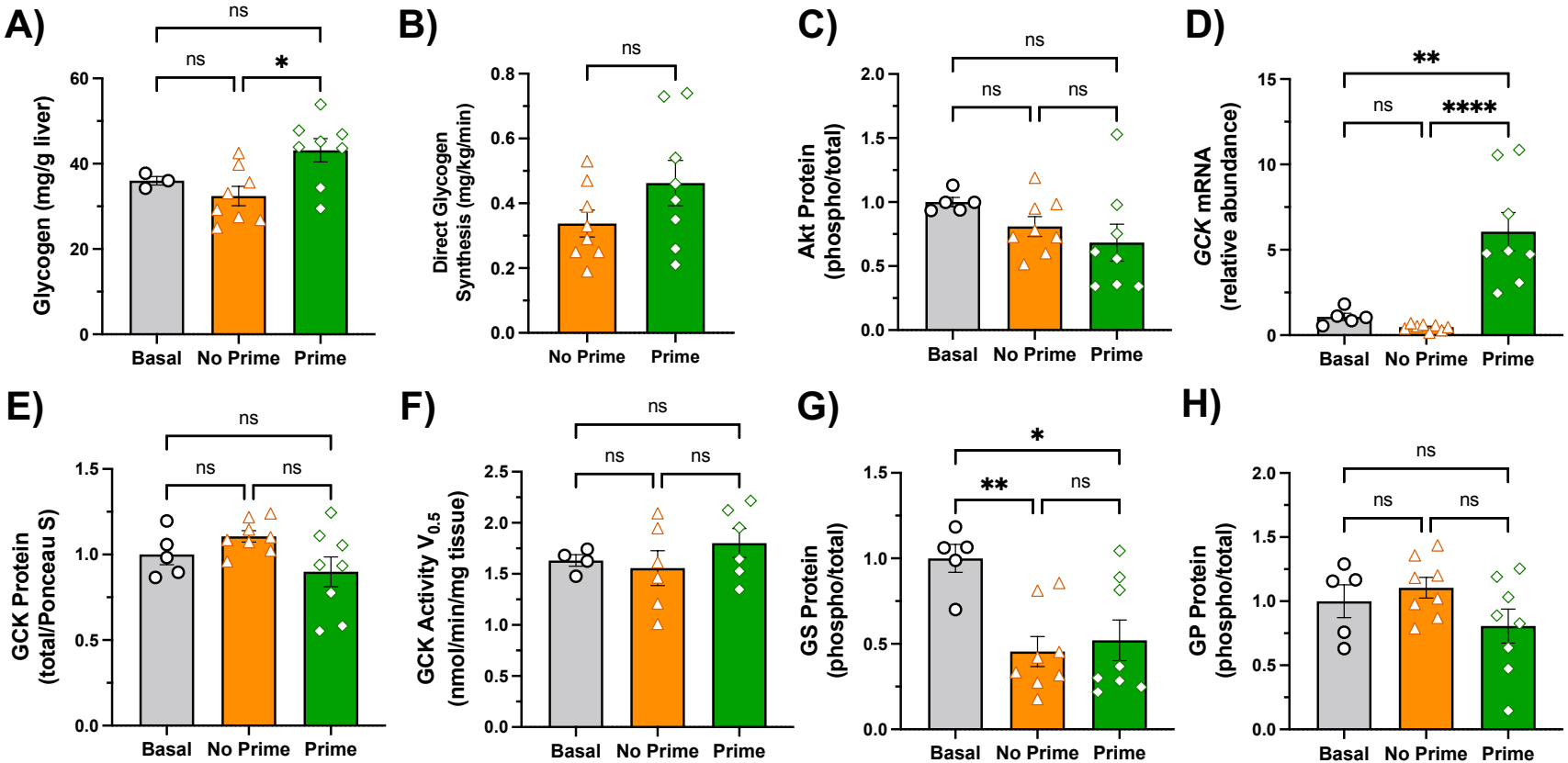

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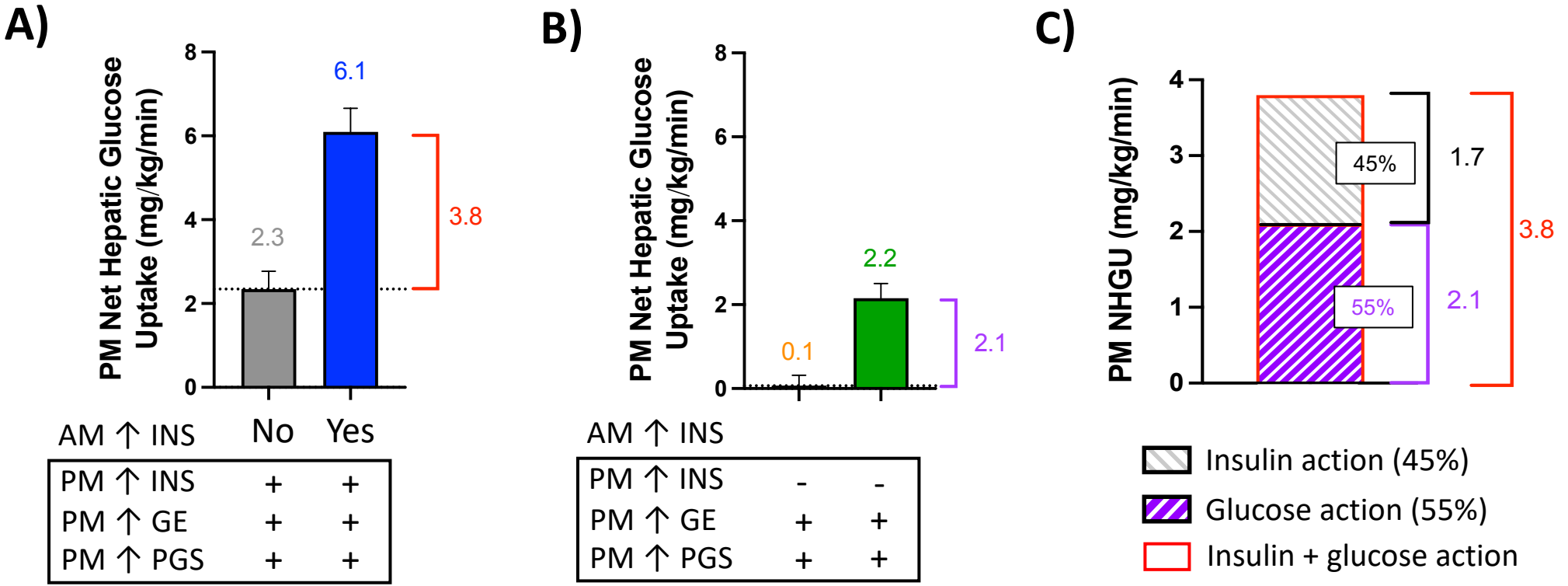

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