Duration of Morning Hyperinsulinemia is Key to the Enhancement of Hepatic Glucose Uptake and Glycogen Storage Later in the Day

Hannah L. Waterman[1], Mary Courtney Moore[1], Marta S. Smith[1], Ben Farmer[1], Melanie Scott[1], Dale S. Edgerton[1], Alan D. Cherrington[1]

[1]Department of Molecular Physiology & Biophysics, Vanderbilt University School of Medicine

New and noteworthy

Morning insulin exposure primes the liver for increased hepatic glucose uptake and glycogen storage during a subsequent meal. This study addressed whether the pattern and/or duration of meal-associated insulin delivery in the morning influences insulin's ensuing priming effect. We found that in the morning, a smaller amount of insulin delivered over a longer duration is more effective than a larger amount over a shorter duration in improving liver glucose metabolism in the afternoon.

Abstract

The second meal phenomenon refers to the improvement in glucose tolerance seen following a second identical meal. We previously showed that 4 hours of morning (AM) hyperinsulinemia, but not hyperglycemia, enhanced hepatic glucose uptake (HGU) and glycogen storage during an afternoon (PM) hyperinsulinemic hyperglycemic clamp (HIHG). Our current aim was to determine if the duration or pattern of morning hyperinsulinemia is important for the PM response to a HIHG clamp. To determine this, we administered the same total amount of insulin either over 2h in the first half of the morning (Ins2h-A), over 2h in the 2nd half of the morning (Ins2h-B), or over the entire 4h (Ins4h) of the morning. In the 4h PM period, all three groups had 4x basal insulin, 2x basal glycemia, and portal glucose infusion to simulate a meal. During the PM clamp, there was a marked increase in the mean hepatic glucose uptake and hepatic glycogen synthesis in the Ins4h group compared to the Ins2h-A and Ins2h-B groups, despite matched hepatic glucose and insulin loads. Thus, the longer duration (Ins4h) of mild

hyperinsulinemia in the morning seems to be the key to much greater liver glucose uptake during the PM clamp.

Introduction

Individuals who regularly skip breakfast have an increased risk of becoming obese, developing type 2 diabetes, and being diagnosed with cardiovascular disease (1-7). While there is an association between breakfast consumption and improved metabolic health, the nutritional composition of the first meal of the day is an important factor to consider when assessing the positive health benefits associated with breakfast (8-11). Specifically, it has been shown that people who consume breakfast meals rich in fibrous whole grains, low-fat dairy, nuts, and fruit display a lower BMI and amount of abdominal fat compared to those who consume breakfast meals composed of high-fat dairy products, processed grains, and artificial sugars (12-14). Moreover, dietary patterns that are high in rapidly available carbohydrates versus slower absorbing carbohydrates are correlated with less favorable metabolic outcomes (15, 16).

One of the primary responses to a carbohydrate-rich meal is the stimulation of insulin secretion, which acts to bring circulating glucose back to basal levels. Meals that are rich in simple carbohydrates, such as refined sugars and processed foods, can lead to a short-lived episode of hyperinsulinemia. Both insulin and glucose levels begin to rise immediately after consuming rapidly absorbed carbohydrates, reaching a peak within 30 minutes to an hour, and remain elevated for only two hours after consumption (17, 18). In contrast, meals that are higher in complex carbohydrates tend to result in a more prolonged, lower bout of hyperinsulinemia (19-22). Furthermore, the level of

hyperinsulinemia is substantially greater after absorbing simple carbohydrates versus complex carbohydrates. For example, one study showed that raw starch ingestion resulted in 35-65% lower insulin response when compared to glucose or sucrose ingestion (23). This glycemic response is worse in individuals with type 2 diabetes due to an insufficient secretion of insulin following ingestion of a carbohydrate, leading to abnormally high postprandial glucose levels (24-26). Thus, it is possible to influence the duration and extent of morning hyperinsulinemia by adjusting meal composition and size.

Human studies have shown that despite being fed an identical meal at breakfast and lunch, glucose and insulin excursions were markedly reduced in response to a second meal. This phenomenon, otherwise known as the “second meal phenomenon” or “Staub-Traugott effect”, has been observed in both healthy individuals and those with diabetes, although some studies suggest that it is abnormal in the latter (27-31). The underlying mechanism explaining this response has yet to be fully uncovered. In previous studies, our lab showed that morning hyperinsulinemia per se is a critical component in priming the liver to increase hepatic glucose uptake and glycogen storage later in the day (32). It was not clear, however, how the duration or kinetics of the rise in meal-associated morning insulin influenced the priming effect. These questions are addressed in the current report and are particularly important for individuals with diabetes, as those who utilize insulin therapy for diabetes management face an elevated risk of experiencing bouts of low blood sugar due to discrepancies in insulin delivery (33, 34). Thus, learning how to maximize the morning priming effect using lifestyle interventions, such as changing meal composition and/or adjusting the amount

of insulin given with each meal in individuals with diabetes, may be an effective way to improve blood sugar regulation.

Materials and Methods

Animal care and surgical procedures

Experiments were conducted on both male and female adult mongrel dogs (22.3±0.6 kg) purchased from a USDA-licensed vendor and housed and cared for according to the standards published by the American Association for the Accreditation of Laboratory Animal Care. The dogs were fed a chow and meat diet (46% carbohydrate, 34% protein, 14.5% fat, and 5.5% fiber). Approximately 2 weeks before being studied, each dog underwent a laparotomy during which catheters were inserted into the left common hepatic vein, the hepatic portal vein, and the femoral artery, and infusion catheters into the splenic vein, jejunal vein, and inferior vena cava (IVC) and buried in a subcutaneous pocket (35, 36). Ultrasonic blood flow probes were placed around the hepatic artery and the portal vein. The experimental protocol was approved by the Vanderbilt IACUC. All dogs were fasted for 18 hours prior to being studied. Only dogs that consumed at least 75% of their last meal, had a leukocyte count <18,000 mm^3, and a hematocrit >34 were studied. The total volume of blood withdrawn was <20% of the animal's blood volume and there was no significant decrease in hematocrit during any of the studies.

Experimental Design

Morning (AM) Clamp*.* The experimental protocol consisted of two clamp periods **(Fig. 1).** At the beginning of each experiment, the catheters and flow probes were

removed from their subcutaneous pockets under local anesthesia. Animals were placed in a Pavlov harness for the remainder of the experimental period. Angiocaths were inserted into the cephalic veins for peripheral infusions. Subsequently, the dogs underwent an AM hyperinsulinemic-euglycemic clamp from 0-120 min, 120-240 min, or 0-240 min (Ins2h-A, Ins2h-B, or Ins4h; *n*=6/group). Thus, the AM clamp began at the same time for the Ins4h group and the Ins2h-A group, and 2 hours later for the Ins2h-B group **(Fig. 1)**. Somatostatin was infused into the inferior vena cava (IVC; 0.8 µg/kg/min) to inhibit endogenous pancreatic hormone secretion. Glucagon was replaced intraportally (0.57 ng/kg/min) to maintain its basal levels. The insulin infusion rates used in the Ins4h group were selected to mimic the rise in endogenous insulin secretion previously observed during a 4h AM duodenal glucose infusion (2.1 mU/kg/min [0-30 min], 2.4 mU/kg/min [30-60 min], and 1.5 mU/kg/min [60-240 min]) (32, 37). These rates were doubled and infused over 2h (4.2 mU/kg/min [0-15 and 120-135 min], 4.8 mU/kg/min [15-30 and 135-150 min], and 3.0 mU/kg/min [30-120 and 150-240 min]) in the Ins2h-A and Ins2h-B groups, respectively, to match the total amount of insulin being infused in the 4h group (405 mU/kg total insulin over the AM clamp period). Plasma insulin levels remain within a physiologic range with these rates (38, 39). 50% dextrose was infused into a peripheral vein as needed to maintain euglycemia. Heparinized saline was infused into the femoral artery catheter during the study to replace fluids. At the end of the AM clamp, all hormone infusions ended. Glucose was infused into the IVC as necessary until the animals were able to maintain euglycemia.

Non-clamp Period. In all groups, a primed, continuous infusion of [3-^{3}H]-glucose (38 µCi prime and 0.38 µCi/min continuous rate) was administered via a peripheral vein

beginning at 240 min. After a 90-minute tracer equilibration period, blood samples were collected from the arterial, portal, and hepatic sampling catheters at 330, 345, and 360 min to allow assessment of glucose kinetics before the PM clamp.

Afternoon (PM) Clamp. All groups underwent a PM hyperinsulinemic-hyperglycemic clamp from 360 to 600 min. Somatostatin was infused as described above, while glucagon (basal) and insulin (4x basal; 1.2 mU/kg/min) were infused intraportally. 20% dextrose was infused into the hepatic portal circulation (4 mg/kg/min) to mimic physiologic hyperglycemia in the portal vein after consumption of a meal. A primed, continuous infusion of 50% dextrose was administered via a peripheral vein to create and maintain hyperglycemia, with the infusion rate adjusted as necessary to clamp arterial blood glucose at ~200 mg/dL. Blood samples were collected from the artery, portal vein, and hepatic vein at 15-30 min intervals to allow measurement of hormones and substrates. Arterial plasma glucose levels were monitored every 5 min during the experimental period. After obtaining the final blood sample, dogs were euthanized while the hormone and glucose infusions were ongoing. Hepatic tissue was rapidly collected, flash-frozen in liquid nitrogen, and stored at -80°C.

Analyses

Biochemical Methods. Arterial, hepatic portal vein, and hepatic vein whole blood samples collected during the study were analyzed using a variety of standard methods to obtain data regarding hormone and substrate balance across the liver. The arterial and portal blood samples were collected at the same time, followed by the hepatic venous blood sample. This allowed for the transit time of blood across the liver. Plasma glucose samples were immediately measured upon collection using a GM9

glucose analyzer (Analox Instruments Ltd., Stourbridge, UK). Blood lactate, glycerol, alanine, and non-esterified fatty acids were analyzed using enzymatic spectrophotometric methods **(see supplement).** Plasma 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 measured by radioimmunoassay. All samples were kept in an ice bath during the experiment and subsequently stored at -80°C until assays were performed. For the determination of [3-^{3}H]-glucose, plasma samples were deproteinized and quantified using liquid scintillation counting (40). Hepatic glycogen was quantitatively assessed using the amyloglucosidase method **(see supplement).**

Molecular Analysis. Molecular methods have been optimized to accurately assess enzyme activity of glucokinase (GK), glycogen synthase (GS), and glycogen phosphorylase (GP) through colorimetric and radioisotope techniques, total and phosphorylated protein levels for Akt (Ser473), GS, GP and total GK using Western blotting procedures, and mRNA of GK, G6Pase, and PEPCK by RT-PCR **(see supplement).** Basal liver samples from 3 overnight-fasted dogs maintained on a standard chow diet that had undergone no experimental intervention were utilized for reference purposes.

Calculations. Direct net hepatic glucose balance was calculated using the A-V difference method using the equation

$$NHGB = [HGL_{out}] - [HGL_{in}]$$

where $[HGL_{out}] = [BF_h \times G_h]$ and $[HGL_{in}] = [BF_a \times G_a + BF_p \times G_p]$

HGL_{out} represents the glucose load exiting the liver, and HGL_{in} is the glucose load entering the liver. BF represents blood flow, G represents the blood glucose concentration, and A, P, and H represent the hepatic artery, hepatic portal vein, and hepatic vein. Under conditions of hepatic glucose production, NHGB is positive, whereas it is negative when the liver is in uptake mode. Direct net hepatic balance of other substrates across the liver may be calculated using this method as well. To avoid any errors in calculating NHGB due to possible incomplete mixing of substrates infused into the hepatic portal vein, HGL_{in} was also calculated using an indirect method:

$$HGL_{in}\,(I) = (G_a \times BF_h) + GIR_{po} - GUG$$

Where GIR_{PO} is the intraportal glucose infusion rate and GUG is the uptake of glucose by the gastrointestinal tract. GUG was calculated as

$$GUG = BF_p \times (G_a - G_p)$$

Thus, indirect NHGB = $HGL_{out} - HGL_{in}$ (I). There were no significant differences between direct and indirect net hepatic balance for any of the substrates measured. The data in this report were calculated using the direct method. Hepatic sinusoidal hormones and substrates were determined using the equation for HGL_{in}. The fractional extraction of a hormone or substrate by the liver was calculated as the net hepatic balance ÷ HL_{in}. Unidirectional hepatic glucose uptake was determined by multiplying the fractional extraction of [3-^{3}H]-glucose (which is not subject to portal vein mixing errors since the tracer was infused into a peripheral vein) by HGL_{in}. Non-hepatic

glucose uptake (non-HGU) was determined by subtracting HGU and the change in glucose mass in the blood from the total GIR.

Plasma glucose concentrations were converted to blood concentrations using a conversion factor established previously (41). Net hepatic carbon retention (NHCR), a proxy for glycogen synthesis over time, was calculated as

$$NHCR = NHGU + NHLU + NHAU + NHGlyU - HGO$$

Where NHGU represents net hepatic glucose uptake, NHLU represents net hepatic uptake of lactate, NHAU represents net hepatic uptake of alanine, NHGlyU represents net hepatic uptake of glycerol, and HGO represents hepatic glucose oxidation, which was estimated to be 0.2 mg/kg/min throughout the experiment as determined in earlier studies (42). Lactate, alanine, and glycerol were converted to glucose equivalents before calculating NHCR. 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 (43, 44) . Therefore, NHCR is the sum of the net hepatic balance of glucose and gluconeogenic substrates minus hepatic glucose oxidation. Synthesized PM hepatic glycogen was calculated as [(mean PM NHCR x BW x 240 min) ÷ g liver].

Statistical Analysis. Data are expressed as mean ± SEM. Statistical comparisons within and between groups were carried out using a two-way analysis of variance with repeated measures design. One-way analysis of variance was used for temporal assessment within a single group. Post hoc analysis was performed using the Student-Newman-Keuls multiple comparisons test. All statistics were analyzed using

GraphPad Prism software. Respective areas under the curve (AUCs) were compared using one-way analysis of variance with Tukey's post hoc analysis. A P value <0.05 was considered statistically significant.

Results

AM Clamp Data

Plasma glucose concentrations did not differ between any of the groups before the start of the AM clamp and euglycemia was maintained in all dogs during the AM clamp **(Fig. 2A).** During the first 120 min, arterial insulin levels were significantly greater in the Ins2h-A group in comparison to the Ins4h group **(Fig. 2B).** At 120 min, insulin infusion ceased in the Ins2h-A group and began in the Ins2h-B group. From 120 to 240 min, Ins2h-B achieved similar arterial insulin levels to those in Ins2h-A**.** The calculated areas under the curve for Ins2h-A and Ins2h-B were 2-fold greater than that of Ins4h, with no significant difference between the 2h groups **(Fig. 2C).** The glucose infusion rate (GIR) for each group reached comparable maximum rates, however, the GIR for Ins4h was sustained throughout the entire AM clamp, then decreased, whereas GIR decreased after the end of insulin infusion at 120 min in Ins2h-A and after 240 min in Ins2h-B **(Fig. 2D).** The ΔAUC for GIR was 2-times greater in Ins4h than Ins2h-A or Ins2h, despite the ΔAUC for insulin being half as great in Ins4h **(Fig. 2E)**. The ratio of the AM clamp GIR AUC to the arterial insulin AUC serves as an index of insulin action. This ratio was 4-fold greater in Ins4h than in Ins2h-A and Ins2h-B **(Fig. 3)**. Glucose infusion continued in Ins4h and Ins2h-B after the end of the AM clamp (during the non-clamp period) to maintain euglycemia until exogenous glucose was no longer needed.

PM Clamp Data

During the PM clamp, postprandial-like conditions were created by doubling the blood glucose level **(Fig. 4A, 4B, 4D),** creating a negative arterial-to-portal vein glucose gradient **(Fig. 4C),** and increasing insulin 4-fold **(Fig. 4E, 4F)** using intraportal glucose and insulin infusions**.** Additionally, the arterial and hepatic sinusoidal plasma glucagon concentrations were kept at basal levels throughout the PM clamp in all groups. Elevated cortisol levels are indicative of an increased stress response, resulting in dysregulated glucose homeostasis (45, 46). There were no differences in arterial cortisol levels between groups, and these levels remained low throughout the experiment **(Table 1).**

The total average GIR (portal and peripheral glucose infusion) during the PM clamp trended higher in Ins4h vs. Ins2h-A and Ins2h-B (17.7 ± 2.3 vs. 13.7 ± 2.4 and 13.3 ± 1.2 mg/kg/min, respectively), but this did not reach significance **(Fig. 5A).** The ΔAUC for GIR also tended to be greater in Ins4h vs. Ins2h-A and Ins2h-B (3621 ± 499 vs. 2743 ± 453 and 2655 ± 253 mg/kg/4hr, respectively; **Fig. 5B**). All dogs rapidly switched to a state of hepatic glucose uptake (HGU) at the onset of the PM clamp. HGU was 40% and 30% greater in Ins4h compared to Ins2h-A and Ins2h-B, respectively (ΔAUC for HGU 1393 ± 199 vs. 822 ± 68 and 900 ± 76 mg/kg/4hr, respectively, $P<0.05$) with no difference between the 2h groups **(Fig. 5C, 5D).** Non-hepatic glucose uptake, which under these conditions is primarily attributed to skeletal muscle glucose uptake, was indistinguishable between groups throughout the PM clamp, although there was a tendency for non-HGU to be slightly greater in the Ins4h group **(Fig. 5E, 5F).** Net hepatic carbon retention (NHCR), a proxy for hepatic glycogen storage, was

significantly greater in Ins4h than in Ins2h-A or Ins2h-B (**Fig. 5G,** P<0.013). 75-85% of the glucose entering the liver (HGU) in all groups was stored as glycogen, whereas the other 15-25% was utilized in hepatic glucose oxidation or converted into lactate. The NHCR ΔAUC for Ins2h-A and Ins2h-B were both 63% of the ΔAUC for Ins4h **(Fig. 5H).** At the onset of the PM clamp, net hepatic non-esterified fatty acid, glycerol, and alanine uptake decreased and remained suppressed **(Table 2).** All dogs switched to net hepatic lactate output and remained in that state for the entirety of the PM clamp, with no differences between groups **(Table 2).**

Liver tissue analyses

Compared to basal overnight fasted dogs, Akt phosphorylation was elevated similarly in all groups following the PM clamp **(Fig. 6A).** Glucokinase (GK) mRNA levels were not significantly different between the three groups, although GK transcript levels were greatest in the Ins4h group **(Fig. 6B).** Furthermore, GK protein expression and activity were elevated in all three groups compared to basal in a trend similar to that of GK mRNA levels **(Fig. 6C, 6D).** These data suggest that the increase in PM hepatic glucose uptake in the Ins4h group compared to the 2h groups does not appear to be due to an acute increase in insulin receptor activation or GK transcription, protein levels, or enzyme activity.

When assessing the enzymes involved in glycogen metabolism, all three groups had greater dephosphorylation and thus activation of glycogen synthase (GS) when compared to baseline **(Fig. 6E)**. The greatest GS activity was observed in the Ins4h and Ins2h-A groups when compared to basal, although there was a strong tendency for

Ins2h-B to be lower than Ins4h and Ins2h-A **(**P<0.074, **Fig. 6F)**. Additionally, the Ins2h-B group had significantly greater dephosphorylation and thus deactivation of glycogen phosphorylase (GP) when compared to the other groups **(Fig. 6G).** There were no apparent differences between any of the groups regarding the suppression of GP activity **(Fig. 6H).** When assessing hepatic glycogen synthesis during the PM clamp, the Ins4h group had 31% and 36% greater amounts of synthesized hepatic glycogen than the Ins2h-A group and the Ins2h-B group, respectively **(Fig. 6I).** There was a tendency for the molecular responses in the Ins2h-B group to be somewhat less responsive to the postprandial signals during the PM clamp compared to the Ins4h and Ins2h-A groups, although not statistically significant (except for GP phosphorylation).

Discussion

Although the second meal phenomenon was identified more than a century ago, the factors determining it have remained poorly understood (47). It has been hypothesized that the lower glycemic response observed upon consuming a second glucose load could be due to an enhancement in muscle insulin sensitivity, enhanced insulin secretion, a reduction in the absorption of oral glucose mediated by increased GLP-1 secretion, lower circulating plasma free fatty acid (FFA) levels, or a combination of these factors (27, 29, 48). Our current and previous data, along with that of others, do not support the involvement of any of these factors in the phenomenon (27, 32, 37, 49). Rather, our recent studies determined that the second meal phenomenon was primarily due to enhanced hepatic glucose uptake secondary to 4 hours of elevated insulin exposure in the morning (32). This raises the question of how morning insulin delivery

might be tailored to maximize this response. We sought to address this issue by determining the importance of 1) the duration of AM insulin exposure, 2) the magnitude of AM insulin exposure, and 3) the timing between AM insulin exposure and the PM clamp (mimicking a second meal).

The total amount of insulin delivered in the morning was identical in the Ins4h and both 2h groups, yet the insulin AUCs were twice as great in the 2h groups. The peak insulin levels in both the Ins2h-A and Ins2h-B groups (30 min after the start of the AM clamp) were ~4-fold greater than the peak insulin level in the Ins4h group, despite both 2h groups only having a doubling in the insulin infusion rates compared to the Ins4h group over the 2h infusion period. The capacity for insulin clearance by the kidneys and liver, the main organs responsible for removing insulin from the bloodstream, can become saturated, and this most likely explains the observed differences in insulin levels (50-52). However, it is important to recognize that despite the greater rate of insulin delivery in both 2h groups, the glucose infusion rates required to maintain euglycemia were the same as those seen in the Ins4h group, thus the lower levels of insulin (as seen in the Ins4h group) were apparently sufficient to maximally stimulate glucose uptake in the morning. This suggests that the rate of insulin delivery in the AM clamp for the Ins4h group was optimal for priming the liver for enhanced PM hepatic glucose uptake.

We found that along with enhanced HGU, hepatic glycogen storage was greater during the PM clamp when it followed 4h of AM hyperinsulinemia. We next sought to understand the molecular mediators of these effects. When assessing PM insulin action, a mediator of PM HGU, the duration of AM hyperinsulinemia did not appear to

significantly affect the insulin receptor or downstream signaling in the PM clamp. Akt phosphorylation was elevated similarly in all groups, suggesting that the enhancement in PM HGU in the Ins4h group compared to the 2h groups was not due to increased PM insulin action. Despite differences in hepatic glycogen content, the activation of signals mediating glycogen storage does not appear to be significantly different between Ins4h and both 2h groups. Glycogen synthase (GS) and glycogen phosphorylase (GP) activity and protein levels are rate-limiting steps in hepatic glycogen synthesis and breakdown (53, 54). GS and GP activities were not significantly different between the 4h and 2h groups at the time of tissue collection. Additionally, there were no significant differences in PM GK mRNA, GK protein, or GK activity between the three groups, although all groups were elevated compared to basal. Our current assays are unable to accurately measure GK translocation from the nucleus to the cytoplasm, which could be an important mediator explaining the enhanced PM response in the Ins4h group vs. the 2h groups. In the fasted state, GK is complexed with glucokinase regulatory protein (GKRP) and sequestered in the nucleus in its inactive state. However, in postprandial conditions (such as the high glucose conditions in the PM HIHG clamp), GK quickly dissociates from GKRP and moves to the cytoplasm in its active form (55, 56). It has been shown previously that GK rapidly translocates in a manner that correlates with changes in HGU, supporting the hypothesis that GK translocation could be increased in the Ins4h group compared to the 2h groups (57).

Consequent to GK translocation and activation, elevated levels of glucose-6-phosphate elicit a synergistic effect with glucose in promoting GS activation and GP inactivation through allosteric control and has been suggested to impact cellular

localization of these enzymes, which could, in part, potentially explain the increase in PM hepatic glycogen storage in the Ins4h group (58-60). It is noteworthy to mention that our GS and GP activity assays reflect the phosphorylation state of these enzymes, and do not necessarily take into account the allosteric activation of GS or inactivation of GP by glucose-6-phosphate and glucose that do not result in a phosphorylation event (61). These allosteric effects may further explain the increase in PM HGU and NHCR in Ins4h compared to the 2h groups which is not further supported or explained by differences in GS and GP activities. While there does not appear to be significant molecular changes between the 2h groups aside from GP phosphorylation, it is important to note that shifting the exposure of AM insulin to the second half of the morning (Ins2h-B) seems to have a subtle effect on GK transcription, protein, and activity, which are all trending lower than observed in the Ins4h and Ins2h-A groups. Furthermore, although GS activity is lower in Ins2h-B, GP activity is as well. The ratio of GS activity over GP activity suggests the effects on these enzymes somewhat cancel each other out, which could explain why there does not appear to be an impact on PM hepatic glycogen synthesis in Ins2h-B relative to Ins2h-A. The variance observed in the molecular data makes it difficult to draw any solid conclusions about how the timing between AM insulin exposure and the PM HIHG clamp might be impacting the transcription and function of key enzymes involved in glucose uptake and glycogen metabolism during the PM.

In a study done previously, we found that PM HGU was 2.9 ± 0.2 mg/kg/min on average in dogs that received a 4h morning duodenal saline infusion (mimicking breakfast skipping), and the same 4h PM HIHG clamp as used in this study (37). Although PM HGU was lower in the 2h groups (3.7 ± 0.3, and 4.4 ± 0.4 mg/kg/min in

Ins2h-A and Ins2h-B, respectively) compared to the Ins4h group, it was still enhanced relative to the saline control study. Thus, even though the 2h duration of insulin exposure in the morning did not maximize the afternoon response (as seen in the 4h group, mean PM HGU of 6.3 ± 0.9 mg/kg/min), it did have a modest stimulatory effect. These results are consistent with findings in healthy adults indicating that slow-release carbohydrates, compared with more rapidly absorbed foods consumed at breakfast, improved the response to a standardized lunch (62). In that investigation, the glycemic pattern was mimicked by carefully matched feeding over a 4h period, but insulin responses were not matched throughout the morning period. Thus, those findings do not provide information about the impact of the pattern of the insulin response throughout the morning period, which we show in this report.

In conclusion, the duration of insulin exposure is more important than the magnitude of insulin exposure in regulating hepatic glucose uptake and glycogen synthesis during a second meal. When the same amount of insulin was administered in the morning, 4h of mild hyperinsulinemia had a greater effect on hepatic glucose uptake and glycogen storage in the afternoon than did 2h of greater hyperinsulinemia. Although the rate of insulin infusion was lower in the Ins4h group compared to the 2h groups, the maximum AM GIR was similar in all three groups. It is evident that the liver is highly responsive to low levels of insulin, with the levels attained in the Ins4h group prompting a robust effect on hepatic glucose uptake and storage in the afternoon. The results of this study provide valuable insight into how meal composition and size might be adapted to improve glycemic control and maximize hepatic glycogen stores, which is particularly important for individuals with insulin-dependent diabetes. Delivering a

smaller amount of insulin in the morning over an extended period versus a larger amount of insulin over a shorter period will prompt greater hepatic glucose uptake and glycogen storage at lunchtime and perhaps even later meals. Additionally, having an ample amount of time between meals may be beneficial to achieve increased glucokinase transcription and translation later in the day.

Data Availability. The data generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

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M.S.S, B.F., M.S., and D.S.E. participated in the experiments; B.F. was responsible for surgical preparation and oversight of animal care; 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., and A.D.C. edited and revised the manuscript; All authors approved the final version of the manuscript. 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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Figure Legends

1. **Study design**. Dogs underwent two glucose clamp periods. All groups had a different pattern of insulin delivery for the morning (AM) hyperinsulinemic-euglycemic clamp, but ultimately received the same amount of insulin over a 2h or 4h period (405 mU/kg total insulin). Following the AM clamp, there was a 90-min tracer equilibration period (240 to 330 min) followed by 30 min of sampling under non-clamp conditions (330 to 360). Subsequently, all dogs underwent an afternoon (PM) hyperinsulinemic-hyperglycemic clamp with portal glucose delivery (360 to 600 min). Tissue collection occurred at the end of the PM clamp (600 min). Details can be found in the methods section. IV; intravenous infusion. Portal; portal vein infusion.
2. **AM hyperinsulinemic-euglycemic clamp data**. A vertical line at 120 min marks the end of the Ins2h-A and the start of the Ins2h-B clamp period. Arterial plasma glucose (A) and insulin (B) concentrations, as well as glucose infusion rates (D) required to maintain euglycemia over the course of the 4h morning period, are shown for Ins4h, Ins2h-A, and Ins2h-B; *n*=6/group. Bar graphs indicate the area under the curve (AUC) for arterial insulin concentrations (C) and glucose infusion rates (E) during the

4h morning period in each dog. Data are expressed as mean ± SEM. *P<0.05, **P<0.01 between groups. All comparisons that did not reach statistical significance are denoted ns.

3. **AM insulin action index.** Morning (AM) glucose infusion rate area under the curve expressed over the AM arterial plasma insulin area under the curve. Data are expressed as mean ± SEM. **P<0.01 between groups. ns = not significant.
4. **PM clamp glucose and hormone data.** A vertical line at 360 min marks the start of the afternoon (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), and plasma insulin at the hepatic sinusoids (F) during the last 30 min of the non-clamp and 4h PM clamp period are shown for Ins4h, Ins2h-A, and Ins2h-B; *n*=6/group. All groups were matched during the PM clamp for these parameters. Data are expressed as mean ± SEM.
5. **PM hyperinsulinemic-hyperglycemic clamp glucose uptake.** A vertical line at 360 min marks the start of the afternoon (PM) clamp. Glucose infusion rate (A), hepatic glucose uptake (C), non-hepatic glucose uptake (E), and net hepatic carbon retention (G) are shown over time. Their respective area's under the curve (B, D, F, H) are shown for the non-clamp and PM clamp period for Ins4h, Ins2h-A, and Ins2h-B; *n*=6/group. *P<0.05, **P<0.01 between groups. ns = not significant.
6. **Hepatic liver tissue analyses.** Phosphorylated Akt protein (A), glucokinase (GK) mRNA (B), GK protein (C), GK activity (D), phosphorylated glycogen synthase (GS) protein (E), GS activity (F), phosphorylated glycogen phosphorylase (GP) protein

(G), GP activity (H), and synthesized hepatic glycogen during the afternoon (PM) clamp (I) are shown for basal when applicable (*n=3-4)*, as well as Ins4h, Ins2h-A, and Ins2h-B (*n*=6/group). Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 between groups. All other comparisons not denoted with a P-value are not significant (ns).

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Table 1: Average plasma hormone concentrations during the AM clamp, non-clamp, and PM clamp periods.

Parameter and group	AM Clamp (0-240 min)	Non-Clamp (240-360 min)	PM Clamp (360-600 min)
Arterial Glucagon (pg/mL)			
Ins4h	20.7 ± 1.7	20.4 ± 2.5	19.3 ± 1.7
Ins2h-A	26.4 ± 1.7	25.6 ± 4.2	22.6 ± 2.1
Ins2h-B	31.6 ± 3.0*	29.2 ± 4.3	25.0 ± 2.4
Hepatic Sinusoidal Glucagon (pg/mL)			
Ins4h	*nm*	26.1 ± 3.0	25.4 ± 2.4
Ins2h-A	*nm*	31.7 ± 5.1	31.9 ± 4.2
Ins2h-B	40.1 ± 3.7	36.8 ± 5.4	38.7 ± 3.6
Arterial Cortisol (µg/dL)			
Ins4h	*nm*	0.7 ± 0.2	1.4 ± 0.8
Ins2h-A	*nm*	0.5 ± 0.2	1.1 ± 0.3
Ins2h-B	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.1

Data are represented as mean ± SEM, n=6 per group. *P<0.05 for Ins4h vs. Ins2h-A and Ins4h vs. Ins2h-B, #P<0.05 for Ins2h-A vs. Ins2h-B. Parameters that were not measured are denoted *nm*.

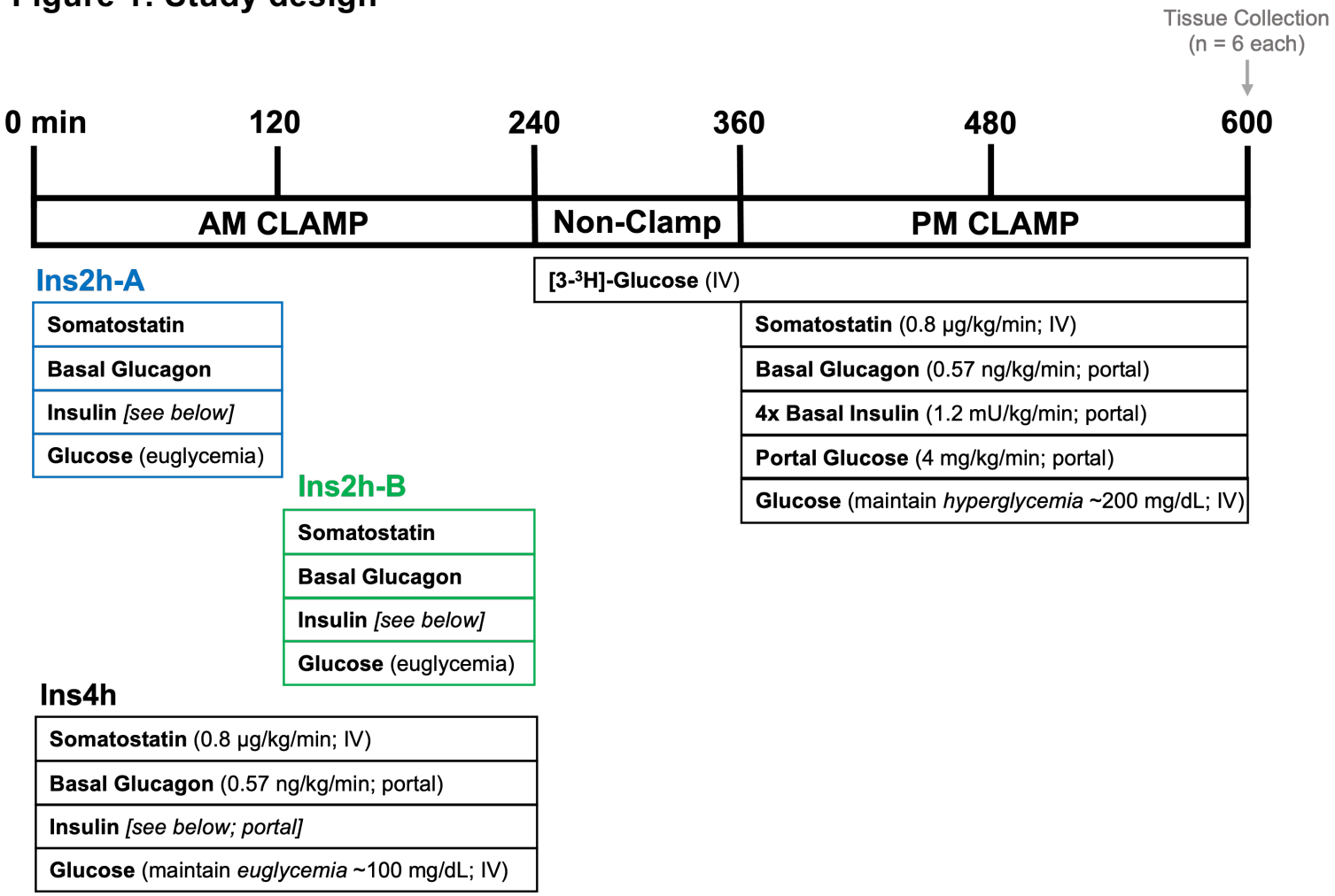
Table 2: PM clamp non-esterified fatty acid and metabolite flux data.

Parameter and group	Non-Clamp	PM Clamp				
	360	420	480	540	600	Group Mean
Arterial Plasma NEFA (μmol/L)						
Ins4h	828±109	113±37	77±30	67±22	46±6	**80±17**
Ins2h-A	960±108	165±44	83±28	71±22	56±19	**128±40**
Ins2h-B	1127±140	102±23	86±18	50±3	86±15*	**100±17**
Net Hepatic NEFA Uptake (μmol/kg/min)						
Ins4h	2.11±0.30	0.42±0.16	0.36±0.15	0.23±0.12	0.26±0.08	**0.26±0.07**
Ins2h-A	1.92±0.17	0.52±0.11#	0.23±0.10	0.30±0.15	0.37±0.14	**0.42±0.11**
Ins2h-B	2.13±0.35	0.31±0.12	0.21±0.09	0.11±0.03	0.17±0.06	**0.20±0.04**
Arterial Blood Glycerol (μmol/L)						
Ins4h	88±12	26±5	22±2	19±4	17±4	**23±3**
Ins2h-A	92±6	37±5	33±6	27±5	26±5	**32±5**
Ins2h-B	123±15	28±5	25±5	29±7	21±4	**27±5**
Net Hepatic Glycerol Uptake (μmol/kg/min)						
Ins4h	2.01±0.35	0.41±0.08	0.34±0.06	0.24±0.07	0.23±0.07	**0.33±0.05**
Ins2h-A	1.97±0.15#	0.79±0.13*	0.56±0.13	0.55±0.14	0.56±0.10*	**0.66±0.12**
Ins2h-B	2.80±0.30	0.42±0.10	0.39±0.10	0.55±0.13	0.28±0.08#	**0.45±0.10**
Arterial Blood Lactate (μmol/L)						
Ins4h	659±169	781±55	656±85	770±103	841±92	**813±77**
Ins2h-A	323±25	848±94	673±84	798±82	824±75	**776±74**
Ins2h-B	380±40	796±82	658±72	710±50	753±68	**711±45**
Net Hepatic Lactate Output (μmol/kg/min)						

Ins4h	-2.8±2.8	16.0±1.8	10.4±2.4	11.6±2.6	10.7±2.4	**12.3±2.1**
Ins2h-A	-3.2±0.8	14.2±3.3	7.9±2.3	7.0±2.0	6.7±1.9	**9.8±2.4**
Ins2h-B	-1.1±1.8	15.8±2.5	9.9±1.7	9.7±1.7	8.8±2.4	**11.3±1.8**
Arterial Blood Alanine (μmol/L)						
Ins4h	265±39	282±25	246±19	234±15	220±17	**250±20**
Ins2h-A	224±15	276±18	244±27	233±30	221±29	**245±27**
Ins2h-B	223±10	243±15	238±19	278±55	217±15	**238±14**
Net Hepatic Alanine Uptake (μmol/kg/min)						
Ins4h	2.41±0.47	2.14±0.22	1.80±0.14	1.76±0.22	1.71±0.26	**1.94±0.21**
Ins2h-A	1.81±0.17	1.29±0.18*****	1.71±0.20	1.56±0.08	1.48±0.16	**1.41±0.10**
Ins2h-B	1.84±0.29	1.45±0.23	1.38±0.13	2.17±0.47	1.14±0.37	**1.50±0.11**

Data are represented as mean ± SEM, n=6 per group. *P<0.05 for Ins4h vs. Ins2h-A and Ins4h vs. Ins2h-B, $^{\#}$P<0.05 for Ins2h-A vs. Ins2h-B.

Figure 1: Study design



Insulin Rates (mU/kg/min):

Ins4h: **2.1** (0-30 min), **2.4** (30-60 min), **1.5** (60-240 min)

Ins2h-A: **4.2** (0-15 min), **4.8** (15-30 min), **3.0** (30-120 min)

Ins2h-B: **4.2** (120-135 min), **4.8** (135-150 min), **3.0** (150-240 min)

- All groups received a total of 405 mU/kg in the AM clamp -

Figure 2: AM hyperinsulinemic-euglycemic clamp data

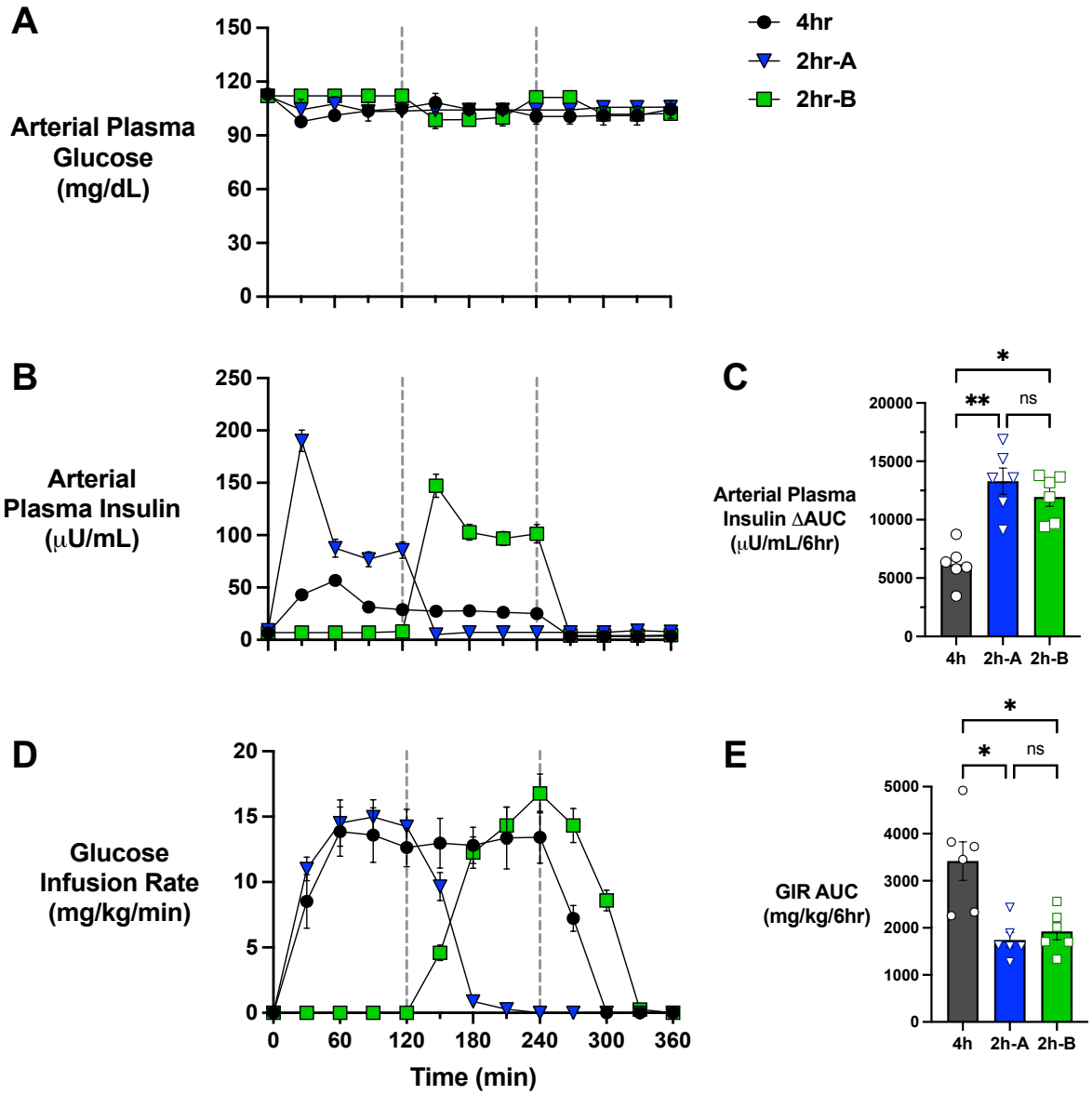


Figure 3: AM insulin action index

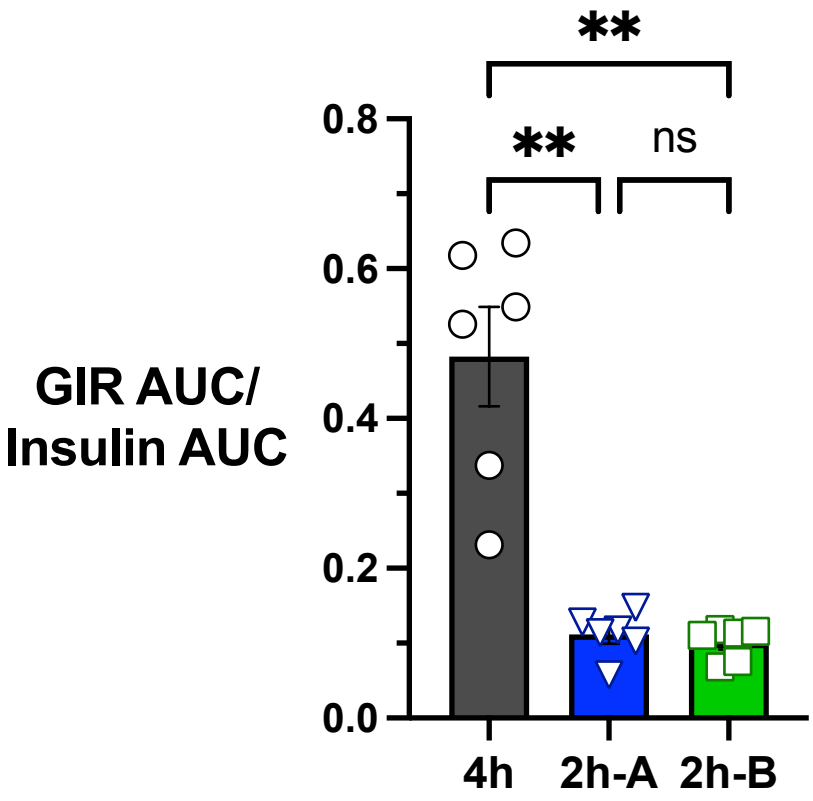


Figure 4: PM clamp glucose and hormone data

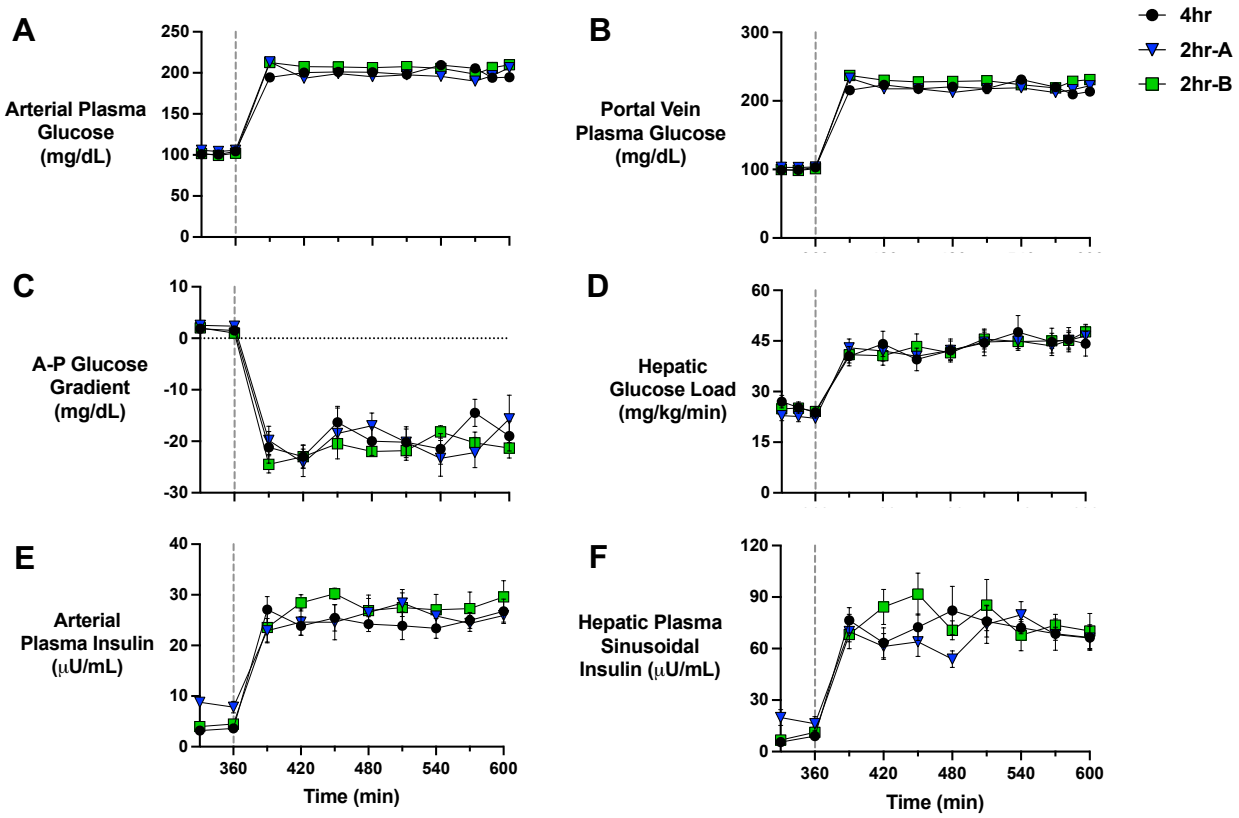


Figure 5: PM hyperinsulinemic-hyperglycemic clamp glucose uptake

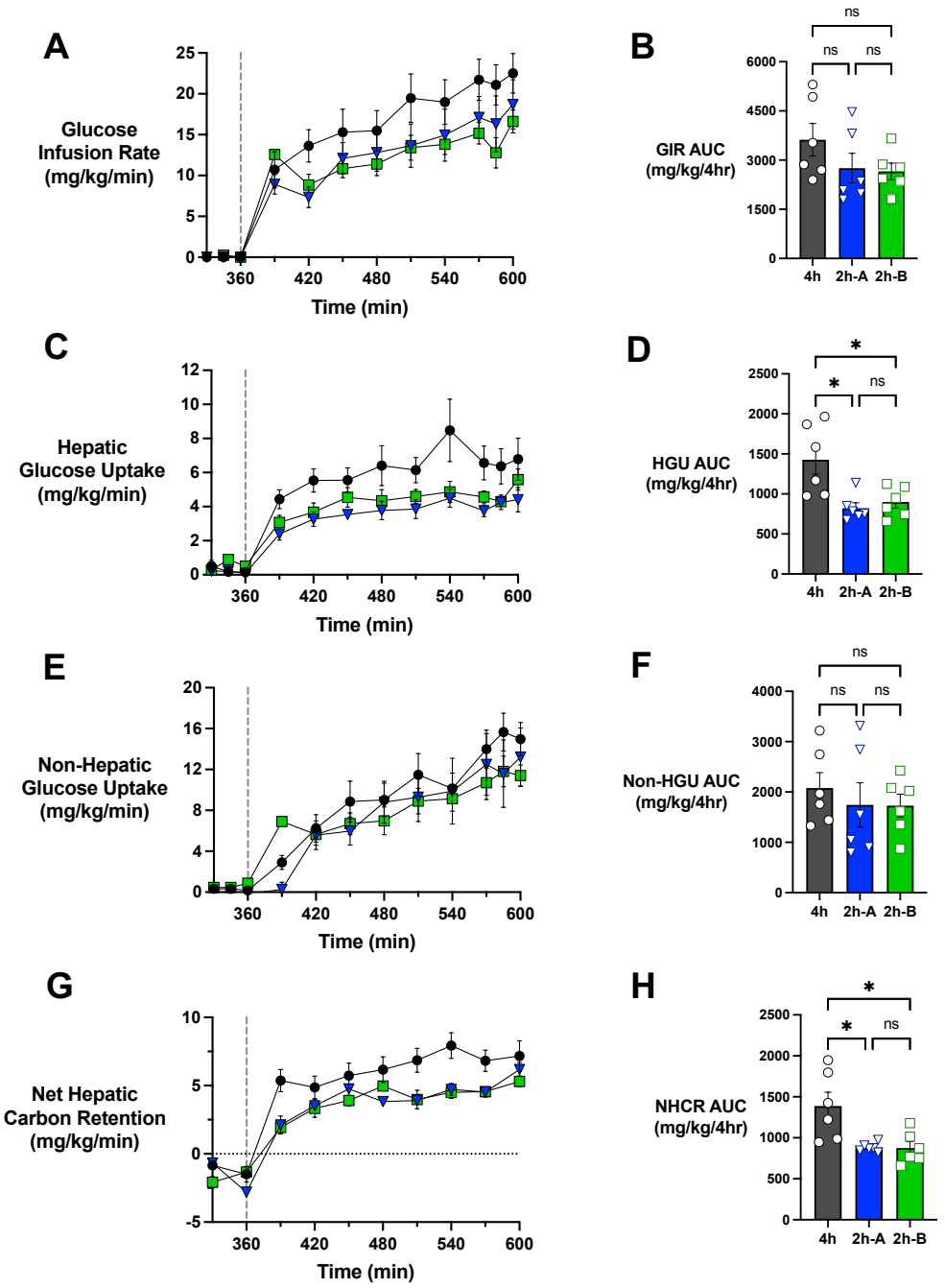
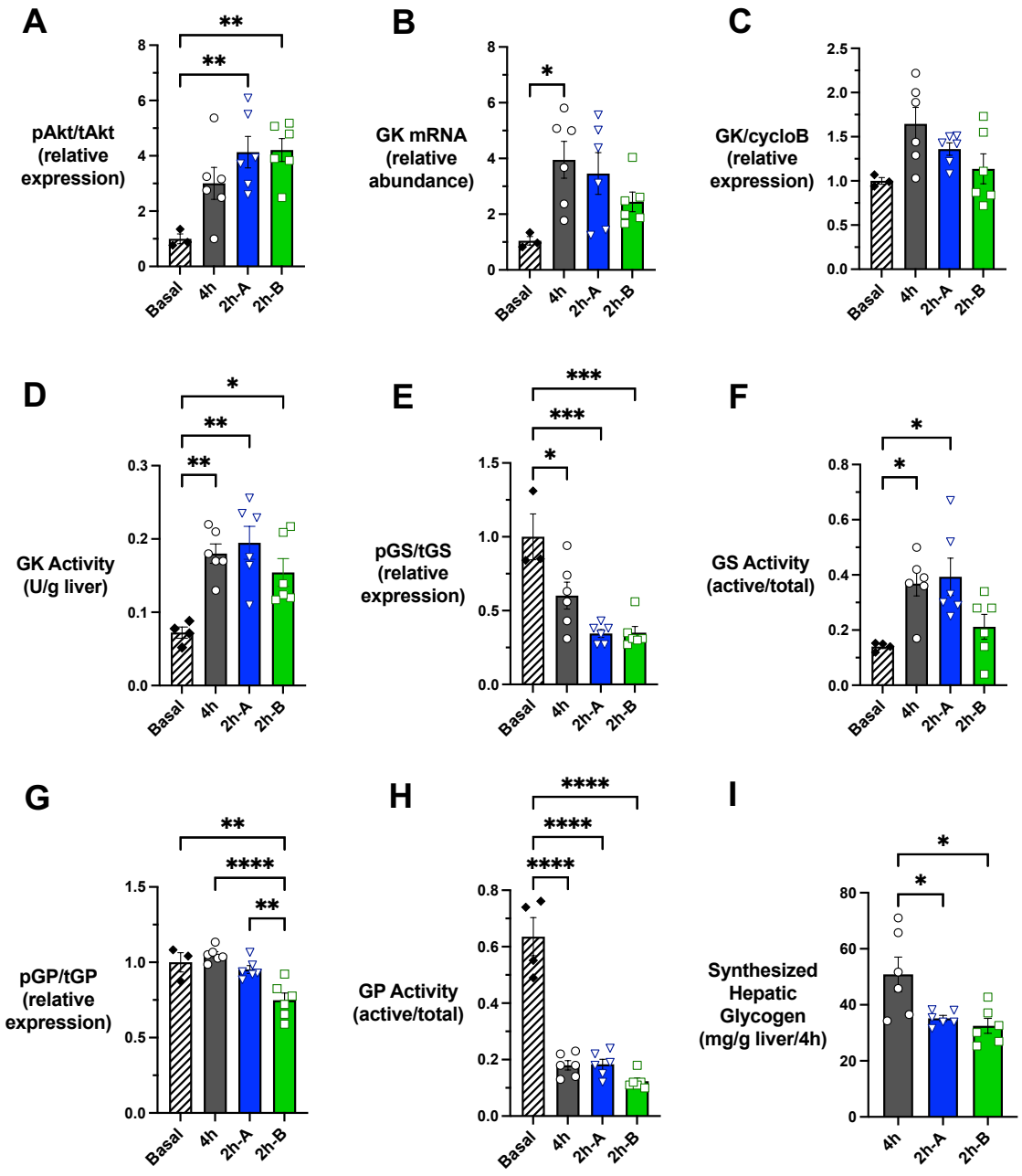



Figure 6: Hepatic liver tissue analyses