The Importance of the Mechanisms by Which Insulin Regulates Meal-Associated Liver Glucose Uptake in the Dog

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METABOLISM

Hepatic glucose uptake (HGU) is critical for maintaining normal postprandial glucose metabolism. Insulin is clearly a key regulator of HGU, but the physiologic mechanisms by which it acts have yet to be established. This study sought to determine the mechanisms by which insulin regulates liver glucose uptake under postprandial-like conditions (hyperinsulinemia, hyperglycemia, and a positive portal vein-to-arterial glucose gradient). Portal vein insulin infusion increased hepatic insulin levels fivefold in healthy dogs. In one group (n = 7), the physiologic response was allowed to fully occur, while in another (n = 7), insulin's indirect hepatic effects, occurring secondary to its actions on adipose tissue, pancreas, and brain, were blocked. This was accomplished by infusing triglyceride (intravenous), glucagon (portal vein), and inhibitors of brain insulin action (intracerebroventricular) to prevent decreases in plasma free fatty acids or glucagon, while blocking increased hypothalamic insulin signaling for 4 h. In contrast to the indirect hepatic effects of insulin, which were previously shown capable of independently generating a half-maximal stimulation of HGU, direct hepatic insulin action was by itself able to fully stimulate HGU. This suggests that under hyperinsulinemic/hyperglycemic conditions insulin's indirect effects are redundant to direct engagement of hepatocyte insulin receptors.

Normal liver function is critical for glucose homeostasis. During fasting, the liver is the body's primary source of glucose, whereas during feeding, it is responsible for storing, processing, and handling a major portion of ingested glucose. Not only can the liver take up as much as one-third of what is consumed in the human (1,2) and dog (3,4), thus equaling muscle glucose disposal, but two-thirds of the typical day is spent with the liver in uptake mode (5). As a result, hepatic glucose uptake (HGU) plays a major role in limiting overall hyperglycemia in healthy individuals. On the other hand, impaired HGU is an important contributor to both fasting and postprandial hyperglycemia in individuals with diabetes (5–11). Development of effective treatments for the defect in HGU requires a better understanding of the mechanisms that regulate this process in vivo.

Upon meal ingestion, HGU is stimulated by the combined effects of *1*) hyperglycemia, *2*) a neural signal that occurs when hepatoportal vein glucose levels are greater than arterial (as is the case when glucose is absorbed from the gut), and *3*) hyperinsulinemia (12). The mechanisms by which insulin regulates HGU under physiologic circumstances remain uncertain, but they likely overlap with those that control hepatic glucose production (HGP). They would include the ability of insulin to directly regulate the liver, initiated by hepatic insulin receptor binding, and insulin's known indirect effects on the liver, mediated by its actions on other tissues, including the adipocyte and the consequent decrease in plasma free fatty acid (FFA), the α-cell and the resulting suppression of glucagon secretion, and the brain via its inhibition of a neural signal originating in the central nervous system. It should be noted that this process is distinct from the "direct" versus "indirect" pathways of carbon flux into liver glycogen (i.e., glycogen derived from glucose in the blood vs. gluconeogenesis), which are also regulated by insulin.

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The mechanisms by which insulin regulates HGU under physiologic conditions have remained unclear, in large part because of the difficulty of measuring HGU in vivo. This parameter is almost impossible to directly measure in the rodent, because of its size, or in man because of the invasive methods required. We previously studied the indirect (nonhepatic) effects of insulin, in isolation, on net HGU in the dog, a model not constrained by the above challenges (13). When arterial (but not portal vein) insulin levels were selectively elevated during a hyperglycemic clamp, we found that insulin's indirect effects on the liver could generate ~ 50% of the response seen when the hormone's direct and indirect effects were both present (13).

How these mechanisms interact is unknown. One possibility is that insulin's direct and indirect effects are additive, with each being responsible for generating a portion of the liver's full response. Alternatively, the indirect effects could be redundant. Because how HGU responds to insulin when insulin is only acting directly is unknown, the aim of the current study was to quantify the impact of insulin's direct effects on the regulation of liver glucose uptake in the absence of its indirect effects on the liver (i.e., secondary to its actions on adipose tissue, pancreas, and brain in overnight-fasted conscious dogs). We hypothesized that HGU would be greatest with insulin's direct and indirect actions all present. The outcome of this study has important implications for diabetes treatment strategies that specifically target insulin to the liver (e.g., with hepatopreferential or oral insulin analogs, intraperitoneal insulin delivery, etc.) (4,14–21). The question is also important because although traditional (peripheral) insulin treatment increases the many clinical risks that are associated with arterial hyperinsulinemia (4,22–24), whether insulin's peripheral effects are required for a full hepatic response remains unclear.

RESEARCH DESIGN AND METHODS

Animals and Surgical Procedures

Studies were performed on 17 conscious 18-h-fasted dogs of either sex (20–23 kg). To provide baseline control data, molecular readouts were compared with liver samples obtained from three healthy overnight-fasted animals. The surgical and animal care facilities met the standards published by the American Association for the Accreditation of Laboratory Animal Care, and diet and housing were provided as previously described (25). The Vanderbilt Institutional Animal Care and Use Committee approved the protocol.

Approximately 17 days before the study, the animals underwent surgery for placement of sampling catheters in a femoral artery and the hepatic portal and hepatic veins, and infusion catheters in the splenic and jejunal veins, which drain into the portal vein (25). Ultrasound flow probes (Transonic Systems, Ithaca, NY) were placed around the hepatic portal vein and the hepatic artery

(25). Ten days before the study, stereotaxic cannulation of the third ventricle was performed as previously described (26–28). All dogs were determined to be healthy before experimentation, as indicated by 1) leukocyte count <18,000/mm³; 2) hematocrit >35%; and 3) good appetite (consuming at least 75% of the daily ration). On the morning of the experiment, the catheters and flow probe leads were exteriorized from their subcutaneous pockets under local anesthesia. Intravenous (i.v.) catheters were also inserted into peripheral leg veins for infusion of glucose, hormones, and radioactive tracer, as necessary.

Experimental Design

Each experiment consisted of a 90-min tracer equilibration period (–120 to –30 min), a 30-min basal sample collection period (–30 to 0 min), and a 4-h experimental period (0–240 min). At –120 min, a primed continuous i.v. infusion of [3-³H]glucose (42 μCi prime and 0.35 μCi/min continuous rate; PerkinElmer) was started to calculate HGU and HGP. At 0 min, glucose was infused into the hepatic portal vein (via the splenic and jejunal catheters) at a constant rate in all groups (4 mg/kg/min) to simulate gut glucose absorption and into a peripheral vein as needed to maintain arterial plasma glucose levels at 200 mg/dL (about twofold basal). Simultaneously, somatostatin (0.8 μg/kg/min; Bachem) was infused i.v. to suppress pancreatic insulin and glucagon secretion, and insulin (Humulin R; Eli Lilly) was infused into the portal vein (1.8 mU/kg/min; about fivefold basal).

In one experimental group, insulin's full effects were in play (direct + indirect [D+I]; n = 7), whereas in the other, insulin's indirect effects were blocked (D-only; n = 7). As used in a previously established protocol (26), in the D-only group, i.v. triglyceride (Intralipid 20%; 0.023 mL/kg/min; Braun) and heparin (0.495 units/kg/min) were infused at 0 min to prevent a decrease in plasma FFA and glycerol, glucagon (GlucaGen; Novo Nordisk) was infused (0.57 ng/kg/min) into the portal vein to maintain its circulating levels at basal. Finally, an insulin receptor antagonist (S961; gift from Novo Nordisk) (29) and PI3K inhibitor (LY294002; Sigma-Aldrich) (30), dissolved in artificial cerebrospinal fluid, were infused into the third ventricle beginning at –90 min, which was shown previously to prevent an increase in hypothalamic insulin signaling, PI3K-mediated activation of hypothalamic K_{ATP} channels (31,32), and the ensuing transcriptional and metabolic effects at the liver (26–28). In the D+I group, FFA levels were allowed to fall naturally, the suppression of glucagon by insulin was mimicked by infusing glucagon intraportally in progressively decreasing amounts (0.54 ng/kg/min during the first 30 min, then a 10% decrease every 30 min thereafter, and activation of hypothalamic insulin signaling was allowed (third ventricle infusion of artificial cerebrospinal fluid) (26–28). The lipid emulsion, used frequently to study the effects of FFA on insulin

action (26,33–38), was composed of essential FFAs, including linoleic, oleic, palmitic, linolenic, and stearic acids, which are the major components of fasting FFAs in the circulation (39).

The insulin infusion rate used in this study was chosen to simulate a modest postprandial-like insulin response (about half the 10-fold rise that can occur after a mixed meal [40]). We reasoned that this rate would give insulin's indirect effects the most likely opportunity to manifest, because insulin's direct effects might overwhelm other mechanisms at higher levels, whereas lower levels might be insufficient to fully engage indirect insulin action. Although postprandial glucose absorption is usually complete within 3 h, the experimental period was extended to 4 h to ensure that all of insulin's acute effects would have sufficient time to manifest.

Data and Analysis Calculations

Plasma glucose was measured using a GM9 glucose analyzer (Analox Instruments Ltd, Amblecote, U.K.), plasma [^{3}H]glucose and nonesterified FFA, and blood lactate, glycerol, and β-hydroxybutyrate concentrations were determined as previously described (25). Glycogen content was measured in liver biopsy specimens taken at the end of the study using the method of Keppler and Decker (41). Plasma insulin (PI-12K, Millipore-Sigma) and glucagon (GL-32K, Millipore-Sigma) were measured by radioimmunoassay. Approximately 20 pg/mL of what is measured by the GL-32K assay is not glucagon (nonspecific cross-reacting material; based on unpublished data from the Vanderbilt University Medical Center Hormone Assay and Analytical Services Core); therefore, the results were corrected to reflect true glucagon levels.

Unidirectional HGU was calculated, as described previously (42), by multiplying the hepatic fractional extraction of [^{3}H]glucose (HFrEx G*) by the hepatic glucose load (HGL; mg/kg/min). HFrEx G* (unitless) was determined by dividing the hepatic [^{3}H]glucose balance by the hepatic [^{3}H]glucose load according to the following equation: $(G*_H \times BF_H - [(G*_A \times BF_A) + (G*_P \times BF_P)])/[(G*_A \times BF_A) + (G*_P \times BF_P)]$, where $G*_A$, $G*_P$, and $G*_H$ represent [^{3}H]glucose values (dpm/mL) in the artery, portal, and hepatic veins, respectively, and BF_A, BF_P, and BF_H represent blood flow (mL/kg/min) in the hepatic artery, portal vein, and liver, respectively. We converted plasma glucose values to blood glucose using previously established conversion factors (43). HGL was calculated according to the following equation: $HGL = G_A \times BF_H + PoG_{inf} - GUG$, where G_A represents the unlabeled blood glucose concentration in the artery (mg/mL), BF_H represents total hepatic blood flow, PoG_{inf} represents the portal glucose infusion rate (mg/kg/min), and GUG represents the uptake of glucose by the gut (mg/kg/min). GUG was calculated as follows: $([G*_A - G*_P]/G*_A) \times (G_A \times BF_P)$, where $([G*_A - G*_P]/G*_A)$ represents the fractional extraction of [^{3}H]glucose across the gut, G_A represents the unlabeled

blood glucose concentration in the artery, and BF_P represents blood flow in the portal vein, respectively. This approach allows HGU to be partitioned from HGP, yielding real-time unidirectional uptake per se (i.e., glucokinase flux) rather than net liver balance.

Net splanchnic glucose balance can be measured in the human, but this challenging procedure requires arterial and hepatic vein blood sampling, measurement of liver blood flow, and reflects the integration of net rates of liver and gut glucose uptake and production. Nuclear MRS can be used to monitor net changes in liver glycogen, which has been used to measure the source of carbon flux into liver glycogen (5), but it does not yield HGU because glucose that is taken up from the blood can be stored as glycogen (often referred to as the "direct pathway" of glycogen synthesis), or it can enter the glycolytic pathway, where it is largely released as lactate or oxidized. Thus, the dog provides a useful model for measuring a parameter that is difficult to assess in other species.

Net hepatic substrate balance (including net HGU) was calculated using the arteriovenous difference method (25). Glucose turnover, used to estimate endogenous glucose production and whole-body R_d, was measured using [3-^{3}H]glucose infusion, based on the GLUTRAN circulatory model of Mari et al. (44) as described elsewhere. Liver glycogen specific activity (SA) was determined by dividing [^{3}H]glycogen (dpm/g liver) by cold glycogen (mg/g liver). Plasma glucose SA was calculated by dividing [^{3}H]glucose (dpm/mL) by cold glucose (mg/mL) in the hepatic sinusoid. The plasma insulin or glucose level entering the liver sinusoids was calculated using arterial and portal vein hormone concentrations and the respective percentage contributions of arterial and portal flow to total hepatic blood flow (25).

Real-time PCR and Western Blot Analysis

RNA isolation, cDNA synthesis, quantitative PCR primers and analysis, and Western blotting procedures were performed using standard procedures as described previously (28,45,46). The nucleotide sequences of dog-specific primers used for glucokinase mRNA quantification were the same as used previously (45). The antibodies for phosphorylated (p)Akt ((S473), total (t)Akt, phosphorylated glycogen synthase (S641), total glycogen synthase, and phosphorylated glycogen synthase kinase-3β (pGSK3β; Ser9) were purchased from Cell Signaling (catalog numbers 927, 9272, 3891, 3893, and 9336, respectively), glucokinase from Santa Cruz (SC-7908), and cyclophilin B from Abcam (ab16045). Test protein bands were quantified using ImageJ software (National Institutes of Health). Liver samples from overnight-fasted animals fed the same diet were used to provide baseline control data. Representative gels are shown in Supplementary Fig. 1.

Statistics

Statistical comparisons were performed with SigmaStat (Systat Software, San Jose, CA) using ANOVA for repeated measures with Student-Newman-Keuls post hoc analysis. Statistical significance was accepted when $P < 0.05$. Data are expressed as mean ± SEM.

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

In response to portal vein insulin infusion, there was close to a fivefold increase in arterial and hepatic insulin levels in both groups. The hepatic sinusoidal insulin level (entering the liver) was about 2.5-fold higher than in the artery, both during the basal period and during the clamp, mimicking the natural physiologic insulin gradient caused by endogenous insulin secretion (Fig. 1A–D). This distribution of insulin, which is essential to understanding the relative importance of direct and indirect insulin action, cannot be created by peripheral insulin infusion (4,47,48). Hepatic insulin load (1.67 ± 0.15 vs. 1.65 ± 0.11 μU/kg/min), net hepatic insulin uptake (0.69 ± 0.10 vs. 0.74 ± 0.05 μU/kg/min), and hepatic insulin fractional extraction (39 ± 3% vs. 45 ± 1%) were not different during the experimental periods of the D+I versus D-only groups, respectively.

In accordance with hyperinsulinemia, hypothalamic insulin signaling was elevated in the D+I group, but the increase in the hypothalamic pAkt-to-tAkt ratio was blocked by the intracerebroventricular infusion of insulin-signaling blockers in the D-only group (1.9 ± 0.2- vs. 1.0 ± 0.1-fold, respectively; $P < 0.05$) (Fig. 2A). Hepatic sinusoidal glucagon levels were similar during the basal period in the D+I and D-only groups (17 ± 2 and 17 ± 3 pg/mL, respectively), but then either fell over time (to 3 ± 1 pg/mL in D+I) or were maintained at baseline (18 ± 3 pg/mL in D-only; $P < 0.05$ between groups) (Fig. 2B) due to the intraportal infusion of glucagon. Arterial FFA levels were similar during the basal period in the D+I and D-only groups (1035 ± 38 and 1198 ± 63 μmol/L, respectively), but then either fell (to 36 ± 8 μmol/L in D+I) or were maintained at baseline (997 ± 72 μmol/L in D-only; $P < 0.05$ between groups) (Fig. 2C) due to the infusion of triglyceride. Likewise, net hepatic FFA uptake was similar in the two groups at baseline (2.3 ± 0.2 and 2.0 ± 0.1 μmol/kg/min, respectively), but then was either nearly completely eliminated (0.1 ± 0.1 μmol/kg/min in D+I), or maintained at baseline (2.0 ± 0.1 μmol/kg/min in D-only; $P < 0.05$ between groups) (Fig. 2D). In contrast, arterial β-hydroxybutyrate levels and net hepatic β-hydroxybutyrate output were suppressed in both groups (Fig. 2E and F). This indicates that it was the direct effect of insulin on the liver, not the lack of FFA substrate, that limited hepatic ketogenesis in the D-only group.

Arterial glucose levels doubled during the experimental period (204 ± 2 and 207 ± 1 mg/dL in the D+I and D-only groups, respectively) (Fig. 3A) due to infusion of glucose into the portal vein and a leg vein. Hepatic glucose loads and portal-to-arterial glucose gradients, important determinants of HGU (12), were similar between groups (Fig. 3B and C). Less glucose was required to maintain the same level of hyperglycemia during the last 90 min of the experiment in the D-only group due to reduced non-HGU, which corresponded to reduced whole-body R_d and reflected a difference in muscle glucose uptake ($P < 0.05$ between groups) (Fig. 3D–F).
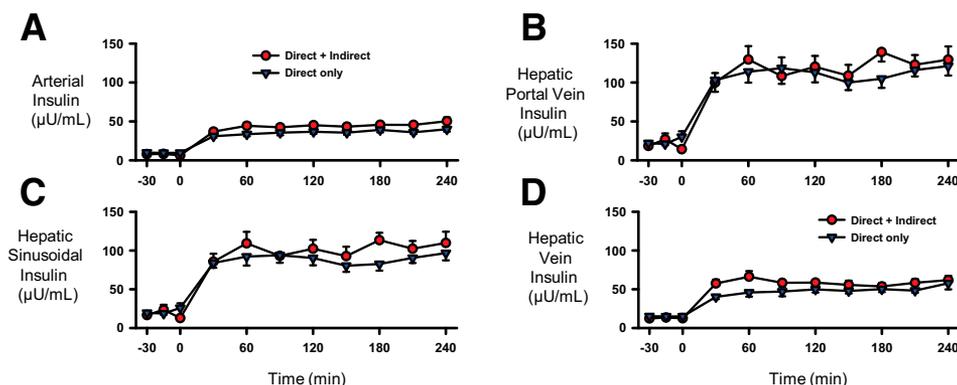
Figure 1 — Insulin levels throughout the body. Arterial (A), hepatic portal vein (B), hepatic sinusoidal (C), and hepatic vein (D) plasma insulin. Overnight-fasted conscious dogs were studied under hyperinsulinemic/hyperglycemic conditions where, after a basal period (–30 to 0 min), insulin was infused into the portal vein to create hyperinsulinemia during the experimental period (0 to 240 min). In one group, the liver was exposed to insulin's full effects (D+I; n = 7), while in the other, only insulin's direct hepatic effects were present (D-only; n = 7) (mean ± SEM). There were no significant differences between groups.
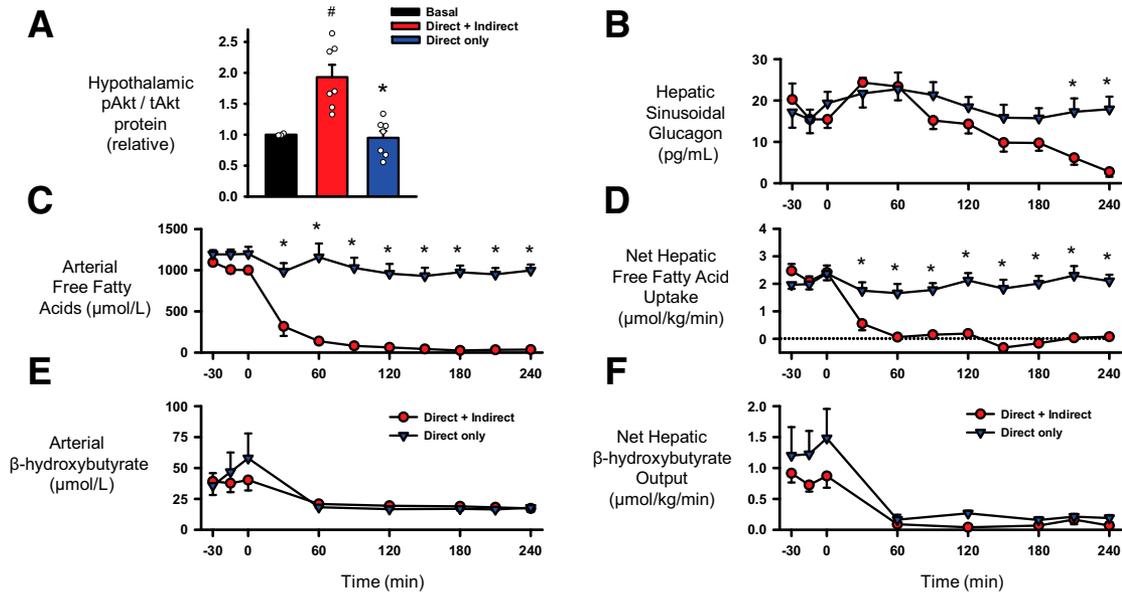
Figure 2—The known indirect mediators of hepatic insulin action were allowed to occur (D+I) or were blocked (D-only). Hypothalamic pAkt-to-tAkt ratio (A), hepatic sinusoidal plasma glucagon levels (B), arterial plasma FFA levels (C) and net hepatic uptake (D), and arterial plasma β-hydroxybutyrate levels (E) and net hepatic β-hydroxybutyrate output (F). Overnight-fasted conscious dogs were studied under hyperinsulinemic/hyperglycemic conditions where, after a basal period (–30 to 0 min), during the experimental period (0 to 240 min) inhibitors of insulin action or artificial cerebrospinal fluid were infused into the third ventricle of the brain and glucagon (portal vein) and triglyceride (peripheral vein) were infused such that the liver was either exposed to insulin's full effects (D+I; $n = 7$) or only insulin's direct effects (D-only; $n = 7$). Liver samples from overnight-fasted animals ($n = 3$) were used to provide baseline control data (basal insulin and glucose) for comparison with tissue taken at the end of each study (mean ± SEM). #$P < 0.05$ vs. basal period; *$P < 0.05$ D+I vs. D-only; unless indicated, there were no significant differences between groups.

We hypothesized that insulin's full effects (direct + indirect) would result in a greater increase in HGU than the direct effect alone, but this was not the case. During the experimental period, HGU was –3.7 ± 0.4 vs. –3.1 ± 0.3 mg/kg/min in D+I and D-only, respectively ($P = 0.3$ between groups) (Fig. 4A). The net hepatic glucose balance was also not different between the groups during this period (–3.4 ± 0.6 vs. –3.0 ± 0.4 mg/kg/min, respectively; $P = 0.6$) (Fig. 4B). Tracer-determined HGP had decreased from basal (2.1 ± 0.1 and 2.3 ± 0.1 mg/kg/min in D+I and D-only, respectively) by 1.5 ± 0.2 and 1.2 ± 0.2 mg/kg/min by the last hour, respectively, and differed slightly between groups at 90 and 240 min ($P < 0.05$) (Fig. 4C). As a secondary method of calculating HGU, HGP was subtracted from the net hepatic glucose balance. Again, this independent, secondary measurement of HGU did not reveal an indirect effect of insulin when insulin's direct effect was concurrently present ($P = 0.7$ between groups) (Fig. 4D).

Glucokinase is a key regulator of HGU (49). In previous studies in the dog, we found that insulin-mediated regulation of glucokinase transcription depends on both the direct hepatic and hypothalamic effects of insulin (27). Similarly, in the current study, glucokinase mRNA levels were markedly increased by hyperinsulinemia ($P < 0.05$ vs. baseline) (Fig. 5A), but tended to be lower (reduced by 35%; $P = 0.06$ between groups) when insulin's indirect

effects were blocked. On the other hand, there was little change in glucokinase protein expression (1.0 ± 0.1- vs. 0.9 ± 0.1-fold basal in the two groups, respectively) (Fig. 5B), probably because there was not adequate time for a change in protein translation to manifest. Liver insulin signaling increased comparably in the two groups, in line with similar hepatic insulin exposure (Akt phosphorylation increased 2.1 ± 0.1- and 2.3 ± 0.1-fold in the D+I and D-only groups, respectively; $P < 0.05$ vs. baseline) (Fig. 5C). In response, hepatic GSK-3β phosphorylation increased (2.3 ± 0.1- and 2.5 ± 0.2-fold basal, respectively; $P < 0.05$ vs. baseline) (Fig. 5D), and glycogen synthase was activated by dephosphorylation (reduced by 75 ± 3% and 50 ± 4%, respectively; $P < 0.05$ between groups and vs. baseline) (Fig. 5E). These changes resulted in an increase in liver glycogen content that paralleled the increase in HGU (36.3 ± 0.5 at basal, increasing to 58.7 ± 2.3 and 57.0 ± 3.0 mg/g liver in the D+I and D-only groups, respectively; $P < 0.05$ vs. baseline) (Fig. 5F). The ratio of [^{3}H]-liver glycogen SA to hepatic sinusoidal [^{3}H]-plasma glucose SA, an index of glycogen formed via HGU rather than gluconeogenic flux to glucose-6-phosphate, did not differ between groups (40 ± 3 vs. 39 ± 5, respectively). Glycogen cycling was assumed to be negligible under the hyperinsulinemic/hyperglycemic study conditions.

Triglyceride infusion during the fat clamp increased glycerol levels and, consequently, net hepatic glycerol
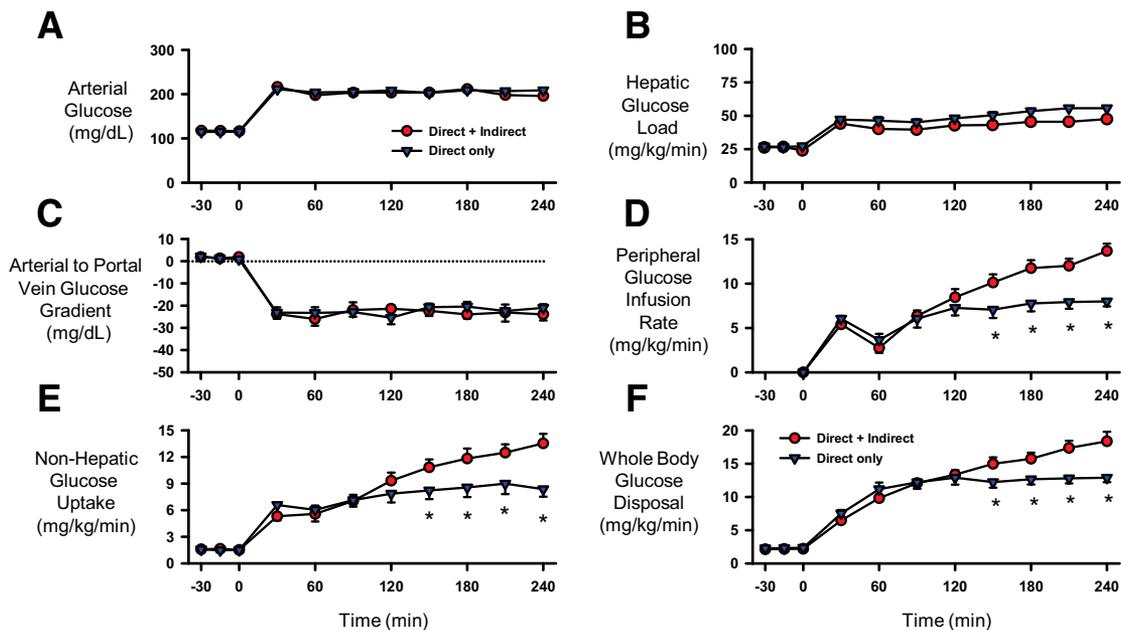
Figure 3—Glucose parameters. Arterial plasma glucose (A), hepatic glucose load (B), arterial-to-portal vein glucose gradient (C), peripheral glucose infusion rate (D), non-HGU (E), and whole-body R_d (F). Overnight-fasted conscious dogs were studied under hyperinsulinemic/hyperglycemic conditions where, after a basal period (–30 to 0 min), glucose was infused into the portal vein (4 mg/kg/min) to create a glucose feeding signal and into a peripheral vein to double the plasma glucose level during the experimental period (0 to 240 min). In one group, the liver was exposed to insulin's full effects (D+I; $n = 7$), while in the other, only insulin's direct hepatic effects were present (D-only; $n = 7$) (mean ± SEM). *$P < 0.05$ D+I vs. D-only; unless indicated, there were no significant differences between groups.

uptake ($P < 0.05$ between groups) (Fig. 6A and B). Alanine is typically the most significant amino acid contributor to gluconeogenic flux. In this study, arterial alanine levels and net hepatic balance were stable and similar between groups (Fig. 6C and D). On the other hand, arterial lactate levels were higher in the D-only group during the last 2 h of the study, while net hepatic lactate output was lower at 60 min ($P < 0.05$ between groups) (Fig. 6E and F). As a result, net hepatic gluconeogenic flux decreased somewhat in both groups during the experimental period
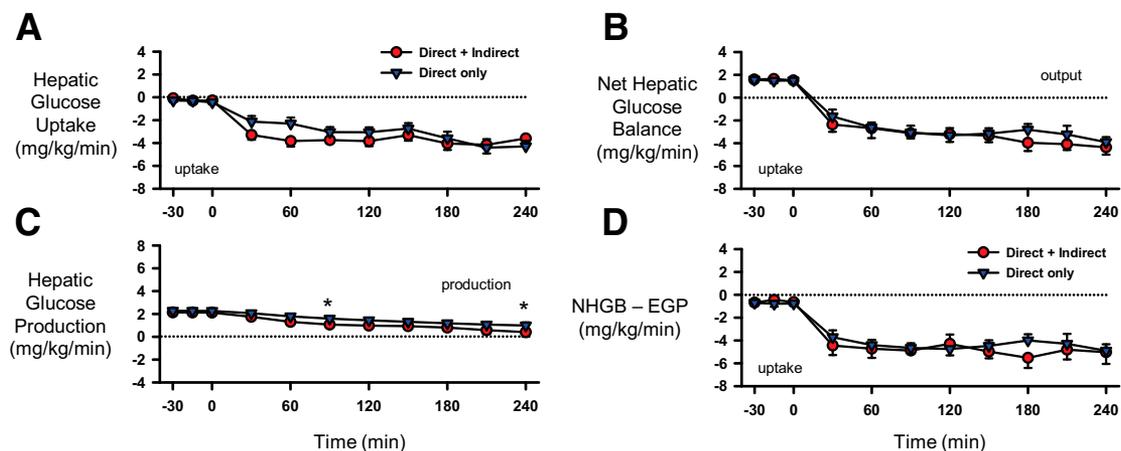
Figure 4—Hepatic glucose metabolism was not affected by the presence or absence of insulin's indirect effects. HGU (A), net hepatic glucose balance (NHGB) (B), HGP (C), and an estimate of HGU calculated by subtracting endogenous glucose production (EGP) from NHGB (D). Overnight-fasted conscious dogs were studied under hyperinsulinemic/hyperglycemic conditions where, after a basal period (–30 to 0 min), animals were either exposed to the full effects of insulin (D+I; $n = 7$) or only to insulin's direct effects (D-only; $n = 7$) during the experimental period (0 to 240 min) (mean ± SEM). *$P < 0.05$ D+I vs. D-only; unless indicated, there were no significant differences between groups.
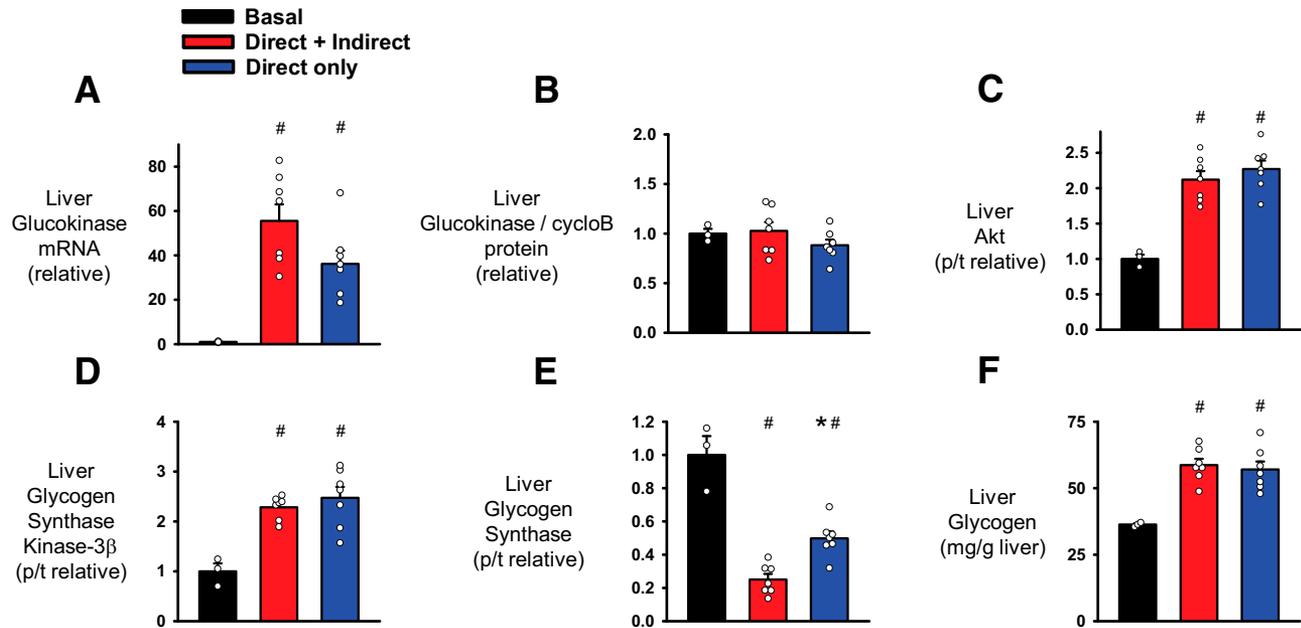
Figure 5—Hepatic insulin signaling. Liver glucokinase mRNA expression (*A*) and protein levels (*B*), p/t Akt (*C*), p/t GSK-3β (*D*), p/t glycogen synthase (*E*), and glycogen (*F*). Overnight-fasted conscious dogs were studied under hyperinsulinemic/hyperglycemic conditions in groups that were either exposed to the full effects of insulin (D+I; *n* = 7) or only to insulin's direct effects (D-only; *n* = 7). Liver samples from overnight-fasted animals (*n* = 3) were used to provide baseline control data (basal insulin and glucose) for comparison with tissue taken at the end of each study (mean ± SEM). *$P < 0.05$ D+I vs. D-only; #$P < 0.05$ vs. basal period; unless indicated, there were no significant differences between groups.

in response to hyperglycemia and hyperinsulinemia, with net glycolysis occurring at 60 min in the D+I group ($P < 0.05$ between groups) (Fig. 6*G*). The magnitude of change in hepatic glycogen metabolism was much greater than the deviations in gluconeogenic or glycolytic fluxes (Fig. 6*G* vs. 6*H*). Fasting net hepatic glycogenolysis switched to net glycogen synthesis in both groups during the clamp, but at the 60-min time point, there was a slightly greater rate of glycogen synthesis in the D-only group ($P < 0.05$ between groups) (Fig. 6*H*). These data demonstrate that most of the glucose taken up by the liver was stored as glycogen, while the indirect effects of insulin only exerted a transient and minor effect to increase net hepatic lactate output at 60 min, thereby briefly decreasing net hepatic glycogen synthesis. This difference was not great enough to exert a meaningful effect on liver glycogen content, however (Fig. 5*F*).

DISCUSSION

Liver glucose disposal plays a critical role in preventing postprandial hyperglycemia in healthy individuals. This process is dysregulated in people with diabetes and contributes to poor glycemic control (5–11). Therefore, the aim of this study was to elucidate the mechanisms by which insulin regulates HGU under hyperinsulinemic/hyperglycemic conditions. Previously, we investigated the effect of a selective increase in arterial insulin (fourfold

rise in the artery, with no increase in insulin at the liver) under hyperglycemic conditions and found that insulin's indirect hepatic effects could only increase net HGU to half the rate seen when the direct and that indirect effects of insulin were both present (13). In contrast, in the current study we found that insulin's indirect effects on the liver were redundant because direct insulin action was capable of generating the full HGU response. Thus, therapies that preferentially target insulin to the liver may have full efficacy while also being safer, compared with traditional (peripheral) insulin therapy, through a reduction in arterial hyperinsulinemia and its associated risks, including hypoglycemia and metabolic and cardiovascular disease (4,22–24).

Insulin induced suppression of plasma FFA, inhibition of glucagon secretion, and increases in hypothalamic insulin signaling have been proposed as indirect mediators of insulin's effects on HGP (26). Here we investigated whether these mechanisms are important determinants of HGU in the normal animal during hyperinsulinemic/hyperglycemic conditions. First, with regard to the effects of FFA on the liver, we previously found that the magnitude of net HGU was inversely associated with the plasma FFA level during basal insulin/hyperglycemic conditions (50). In that study, when plasma FFA levels were nearly completely suppressed by nicotinic acid (a potent inhibitor of lipolysis), net HGU was twice as great as when triglyceride was infused to maintain basal FFA levels.
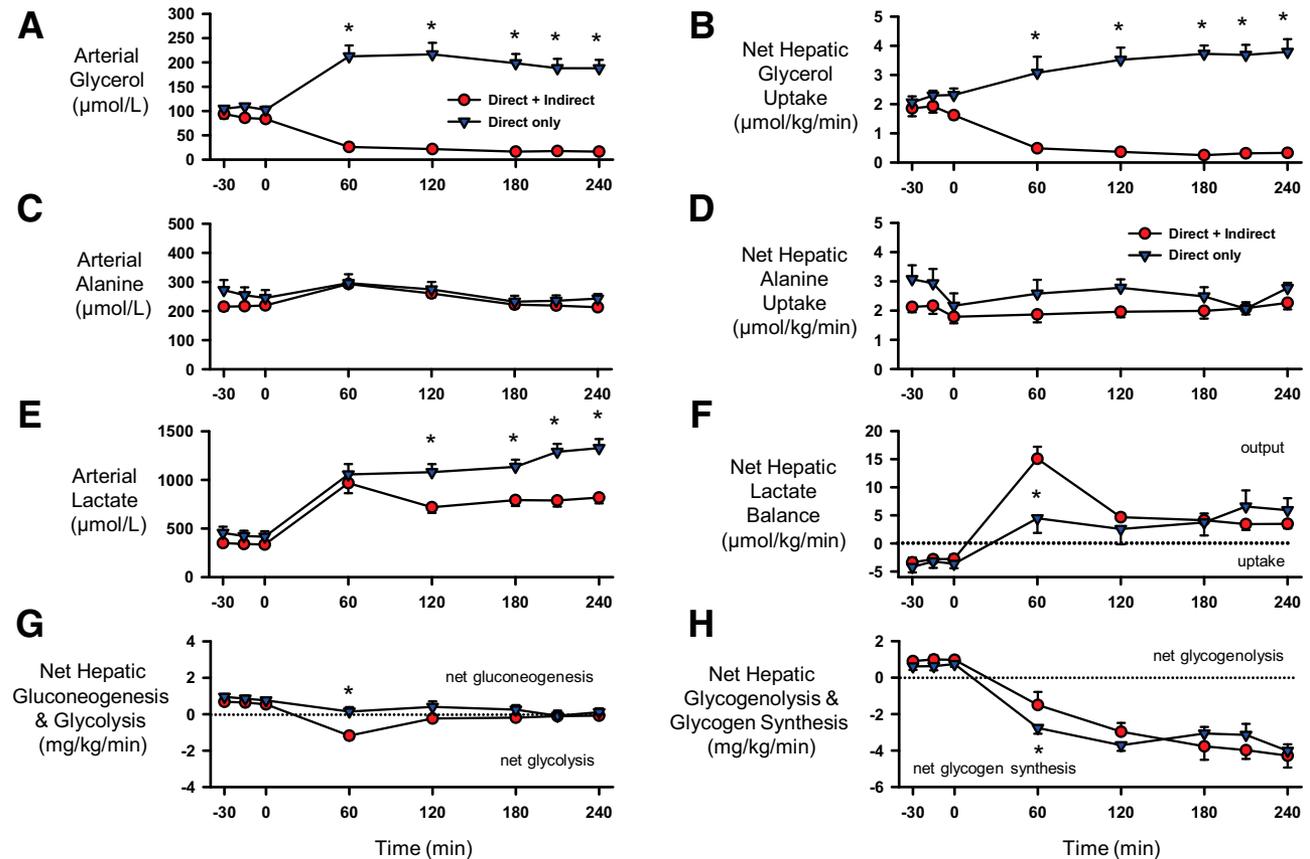
Figure 6—Metabolites and intrahepatic gluconeogenic, glycolytic, and glycogen fluxes. Arterial blood glycerol (*A*), net hepatic glycerol uptake (*B*), arterial blood alanine (*C*), net hepatic alanine uptake (*D*), arterial blood lactate (*E*), net hepatic lactate balance (*F*), net hepatic gluconeogenic and glycolytic fluxes (*G*), and net hepatic glycogenolytic and glycogen synthesis fluxes (*H*). Overnight-fasted conscious dogs were studied under hyperinsulinemic/hyperglycemic conditions where, after a basal period (–30 to 0 min), animals were either exposed to the full effects of insulin (D+I; *n* = 7) or only to insulin's direct effects (D-only; *n* = 7) during the experimental period (0 to 240 min) (mean ± SEM). *$P < 0.05$ D+I vs. D-only; unless indicated, there were no significant differences between groups.

Likewise, in another study (when hepatic insulin was again maintained at basal), suppression of plasma FFA resulted in stimulation of glycolytic flux (net hepatic lactate release increased), while there was a corresponding decrease in net hepatic glucose output (38). It was unclear which components of net hepatic glucose balance (HGP and/or HGU) were affected by FFA in those studies or how a rise in insulin at the liver would have influenced the effects of FFA.

The current study demonstrates that regardless of whether plasma FFA levels decrease or not, insulin's direct effect is capable of generating a full HGU response. At the same time, the results show that FFA can alter intrahepatic carbon flux under hyperinsulinemic/hyperglycemic conditions. When net hepatic FFA uptake was suppressed by insulin (D+I group), there was a greater initial surge in net hepatic lactate output. Loss of available substrate for glycogen synthesis is consistent with the reduced rate of synthesis that was measured at 60 min. Because the effects of insulin on glycolysis and glycogen synthesis were offsetting, however, HGU was

unaffected. Furthermore, this effect was transient, such that by the end of the study, plasma FFA levels had no impact on these rates. Thus, it appears that beyond the first hour, direct activation of glycogen synthase by insulin and glucose was sufficient to direct glucose uptake into glycogen, negating any lasting effect of FFA on these pathways. Clearly, FFA can affect hepatic glucose fluxes when liver insulin levels are low, but as with HGP (26), the effect of FFA on HGU is lost in the face of direct hepatic insulin action.

FFA may have had an impact on other aspects of glucose metabolism. For example, arterial lactate levels were higher in the D-only group during the last 2 h of the study, despite similar rates of net hepatic lactate output. Because muscle lactate production is stimulated by FFA (51), it is possible that an earlier imbalance in muscle lactate release was responsible for this later difference in the circulating level. In addition, the maintenance of plasma FFA levels may have been responsible for impairing non-HGU (e.g., by muscle), which explains the lower glucose infusion rate in the D-only group. This could have

occurred via inhibitory effects of FFA on glucose effectiveness (52) and/or insulin sensitivity (53,54), which can manifest within a few hours (35). Finally, FFAs are known to reduce insulin clearance (55), which may explain the tendency for plasma insulin levels to have been slightly higher in the D+I group (10–20%). If anything, however, higher levels would have promoted greater HGU, thus further strengthening our conclusion that indirect insulin action is not required for normal HGU.

Insulin inhibits glucagon secretion (26), and glucagon opposes insulin's effects on hepatic glycogen synthesis and gluconeogenesis (56). Thus, suppression of glucagon is a second means by which insulin might regulate HGU. Even basal levels of glucagon exert powerful anti-insulin effects (57); therefore, inhibition of glucagon secretion could reasonably cause a reduction in HGU. Previously, we investigated the effect of glucagon on hepatic glucose metabolism under hyperinsulinemic/hyperglycemic conditions (58). In one group, glucagon was infused to increase its plasma level by 21 pg/mL over baseline, while in another group, it was allowed to decrease by 16 pg/mL. Under those conditions, a difference in glucagon of 37 pg/mL increased net hepatic glucose balance (by 2.2 mg/kg/min), primarily via control of HGP; whether it had an effect on HGU, per se, was not clear. In the current study, nearly complete suppression of glucagon secretion (decrease of 14 pg/mL) did not affect the stimulation of HGU but may have had a small effect on HGP.

Finally, hypothalamic insulin action has been proposed to regulate hepatic glucose metabolism via a neural circuit that alters hepatic gene transcription, and ultimately, protein levels (31,32,59). Indeed, when we previously created a selective increase in insulin at the brain (with basal insulin and glucose at the liver), we observed a modest increase in the net HGU after 3 h, with no change in HGP, implying an increase in HGU (28). This effect was associated with increased glucokinase mRNA levels, decreased levels of GSK-3β mRNA and phosphorylation, and reduced glycogen synthase phosphorylation, all of which could favor greater HGU. A postprandial role of central nervous system insulin action in regulating HGU was not established in that study because hepatic insulin and glucose levels were maintained at basal, but it is reasonable to think that HGU might be enhanced by hypothalamic insulin action under hyperinsulinemic/hyperglycemic conditions. This was not the case, however.

Although hepatic glucokinase gene transcription increased after 4 h due to both the direct and indirect effects of insulin on the liver, glucokinase protein levels did not. Therefore, the prompt increase in glucokinase flux (HGU) that occurred in both groups must have been due to the translocation of glucokinase from the nucleus to the cytoplasm, an effect mediated by insulin and glucose (60). Indirect insulin action was also associated with somewhat greater dephosphorylation of glycogen synthase. Despite this, net hepatic glycogen synthesis and liver glycogen accumulation were similar between the groups by the end of the study. Because glycogen synthase is also under allosteric control by glucose-6-phosphate, which increases during hyperglycemia, it is likely that glycogen synthase activity was maximal in both groups. Of note, although indirect insulin action was not responsible for the rapid increase in HGU that occurred, these mechanisms may affect the response of the liver to insulin over a more prolonged time scale, for example, during subsequent meals (61).

Several limitations to our study should be considered. First, triglyceride infusion during the fat clamp increased blood glycerol levels in the D-only group. Of note, however, is that when the corresponding increase in net hepatic glycerol uptake is considered, even if all possible additional glucose-6-phosphate derived from glycerol was released from the liver as glucose rather than stored as glycogen, at most there would have been a 0.1 mg/kg/min underestimation of net HGU in the D-only group. Thus, differences in glycerol should not have affected our findings, but even if they did, it would further strengthen our conclusion. In agreement with this, we previously found that infusion of glycerol to match the rise that occurred during a fat clamp had no measureable impact on net HGU or any related parameter (50).

Second, a 4-h hyperinsulinemic/hyperglycemic clamp with insulin and glucose administered into the hepatoportal vein approximates, but does not fully replicate, the postprandial state, where, during a mixed meal, amino acids and fats are also ingested and absorbed at variable rates over several hours. Clamp studies are often considered the gold standard for studying physiologic variables in isolation, with somatostatin used to precisely control pancreatic hormone concentrations. This method cannot be used during meal studies, however, because somatostatin inhibits gut motility and slows nutrient absorption. Thus, while it is possible to control insulin levels without somatostatin during a euglycemic clamp (because exogenous insulin shuts down endogenous secretion when glucose is basal [26]), studying postprandial glucose metabolism without this tool is much more difficult. Of note, mealtime amino acid ingestion stimulates glucagon secretion, although insulin is still likely to limit the relative extent of this rise. Future studies that take into account additional parameters are needed, including the liver's response to elevated amino acids in the portal vein as well as new aspects of postprandial glucagon physiology that have recently come to light (62).

Third, the route by which insulin is administered largely determines which of several mechanisms it uses to regulate hepatic glucose metabolism. For example, when insulin is delivered or secreted into the portal vein, the current study and others have demonstrated that insulin's direct inhibitory effects are dominant (27,48,63). On the other hand, when insulin is delivered via a peripheral route (e.g., subcutaneous, i.v., or inhalation), such as in a

patient with type 1 diabetes, its indirect effects take on more importance (4,38,47). Furthermore, it remains unknown whether direct insulin action is sufficient to overcome the impairment in HGU caused by a defect in glucokinase in patients with type 2 diabetes (11). Future studies will be necessary to determine how the normal physiology investigated here translates to pathologic regulation of the liver and to new treatment strategies. In addition, although glucoregulation is very similar in the dog and human, it will be important to verify our findings in man.

In summary, the current study demonstrates that under hyperinsulinemic/hyperglycemic conditions, when direct hepatic insulin action is engaged, the suppression of FFA and glucagon and the activation of hypothalamic insulin signaling are not required for normal stimulation of HGU. While insulin's indirect mechanisms are clearly able to independently increase HGU (13), these effects are redundant to insulin's direct effect rather than additive. Thus, this study suggests that insulin engineered to preferentially but not selectively target the liver, despite being delivered into the periphery, should produce a nearly normal hepatic glucose response during feeding. Given the significant risks of hypoglycemia and metabolic disease that are associated with the hyperinsulinemia resulting from peripheral insulin delivery, the development of such approaches seems warranted.

Duality of Interest. A.D.C. has the following potential conflicts of interests: Abvance, Biocon, Diakard/Diabetica, Fractyl, Metavention, Novo Nordisk, Sekkei Bio, Sensulin Laboratories, LLC, Thetis Pharmaceuticals, LLC, and vTv Therapeutics. No other potential conflicts of interest relevant to this article were reported.

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