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Pulsatile Portal Vein Insulin Delivery Enhances Hepatic Insulin Action and Signaling

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Insulin is secreted as discrete insulin secretory bursts at ~5-min intervals into the hepatic portal vein, these pulses being attenuated early in the development of type 1 and type 2 diabetes mellitus (T2DM). Intraportal insulin infusions (pulsatile, constant, or reproducing that in T2DM) indicated that the pattern of pulsatile insulin secretion delivered via the portal vein is important for hepatic insulin action and, therefore, presumably for hepatic insulin signaling. To test this, we examined hepatic insulin signaling in rat livers exposed to the same three patterns of portal vein insulin delivery by use of sequential liver biopsies in anesthetized rats. Intraportal delivery of insulin in a constant versus pulsatile pattern led to delayed and impaired activation of hepatic insulin receptor substrate (IRS)-1 and IRS-2 signaling, impaired activation of downstream insulin signaling effector molecules AKT and Foxo1, and decreased expression of glucokinase (*Gck*). We further established that hepatic *Gck* expression is decreased in the HIP rat model of T2DM, a defect that correlated with a progressive defect of pulsatile insulin secretion. We conclude that the physiological pulsatile pattern of insulin delivery is important in hepatic insulin signaling and glycemic control. Hepatic insulin resistance in diabetes is likely in part due to impaired pulsatile insulin secretion. ***Diabetes*** **61:2269–2279, 2012**

Fasting hyperglycemia in both type 1 and type 2 diabetes mellitus (T1DM and T2DM, respectively) is due to increased hepatic glucose release as a result of insufficient insulin secretion in the context of relative hepatic insulin resistance (1,2). Defective insulin secretion and insulin resistance both develop early in the evolution of T1DM and T2DM (3–5). Hepatic insulin resistance coincides with impaired insulin secretion and precedes diabetes onset in animal models of T2DM with progressive β-cell loss (6,7). Likewise, hepatic insulin resistance develops with β-cell loss in animal models of T1DM (8,9). This raises the question, does β-cell failure contribute to hepatic insulin resistance?

Insulin is secreted in coordinate secretory bursts into the hepatic portal vein with a periodicity of ~5 min (10,11). Insulin secretion is regulated by the magnitude of insulin pulses (12,13). Thus, hepatocytes are exposed to an oscillating insulin concentration wave front with an amplitude of ~0.5–1.0 nmol/L in the fasting state and increasing to ~5.0 nmol/L after meal ingestion (11,12). The vascular anatomy of hepatic sinusoids permits direct exposure of hepatocytes to these insulin oscillations.

Since pulsatile insulin delivery in T1DM and T2DM is impaired (14–16), defective pulsatile insulin delivery may contribute to diminished hepatic insulin signaling and hepatic insulin resistance in diabetes. While several studies have approached this question (11,17–21), none have delivered insulin into the portal vein to reproduce the insulin concentration oscillations and timing present in vivo. To address this, we developed an intraportal infusion protocol in conscious dogs that reproduced the in vivo pulsatile insulin concentration profile in the fasting state (10,11). We then compared this with the same insulin infusion rate delivered as a constant infusion and with an insulin infusion profile that recapitulated defective pulsatile insulin secretion present in T2DM (15). We established first in the dog and then confirmed in the rat that physiological insulin pulses delivered into the portal vein enhance insulin action. We then tested the hypothesis that the mechanism of this was greater efficacy of the pulsatile mode of insulin delivery on hepatic insulin signaling and gene expression.

RESEARCH DESIGN AND METHODS

Canine studies

Study protocol. The institutional animal care and use committee at the University of California Los Angeles approved all dog studies. Five mongrel dogs aged ~1–3 years and weighing 20–24 kg were used in the current studies. Animal care and catheter implantation were performed as previously described (10). Dogs were studied on three separate occasions after a 12-h fast. On each occasion, dogs were placed in a laboratory sling and endogenous insulin secretion was ablated by a 0.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ somatostatin infusion (somatostatin-14; Bachem, Torrance, CA) with basal replacement of glucagon at 0.65 $ng \cdot kg^{-1} \cdot min^{-1}$ (Glucagen; Bedford Laboratories, Bedford, OH) given via foreleg infusion catheter throughout the study (0–300 min). A primed continuous infusion of [6,6-$^2H_2$]glucose was given at a rate of 3 mg/kg/h to trace glucose turnover. The insulin infusion rate required to maintain fasting glucose concentrations in each dog when insulin was delivered via the portal vein in the normal pulsatile fashion was determined for each dog in a pilot study prior to the three study protocols.

For protocol 1 (pulsatile insulin delivery), insulin was infused at the rate previously established in each dog (range 0.9–2.0, mean 1.2 $pmol \cdot kg^{-1} \cdot min^{-1}$) with 70% as 1-min pulses at 6-min intervals and 30% as a constant basal insulin infusion from 0 to 300 min. For protocol 2 (constant insulin delivery), the same total insulin infusion rate for each dog was administered but 100% as a constant infusion. For protocol 3 (T2DM pulsatile infusion), insulin infusion was comparable with protocol 1 except that the magnitude of the insulin pulses was decreased by 50%, reproducing the pattern present in T2DM (15). The mean insulin infusion rate for the T2DM protocol equaled 0.81 $pmol \cdot kg^{-1} \cdot min^{-1}$ (0–300 min). Blood was sampled at 10- to 30-min intervals (0–300 min) from the foreleg venous catheter for measurement of glucose, insulin, C-peptide, and glucagon concentrations. Blood was sampled at 1-min intervals from the portal

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See accompanying commentary, p. 2228.

vein catheter for 40 min (140–180 min) for measurement of insulin concentrations to permit evaluation of the efficacy of the three mesenteric vein insulin infusion profiles in accomplishing the desired portal vein insulin profiles (Supplementary Fig. 1).

Rat studies. More detailed methods for the rat studies are provided in the Supplementary Data online. The institutional animal care and use committee at the University of California Los Angeles approved all rat studies. A total of 71 Sprague-Dawley male rats aged 5 months had catheters placed as described (22). The efficacy of pulsatile insulin delivery on glycemia (protocol 1), insulin sensitivity (protocol 2), and hepatic insulin signaling (protocols 3–5) were then evaluated as follows. In protocol 1, conscious rats were studied, in a manner comparable with that undertaken in dogs, by ablation of endogenous insulin secretion with somatostatin and replacement of basal glucagon (0.65 ng $\cdot$ kg^{-1} $\cdot$ min^{-1}) and insulin (5 pmol $\cdot$ kg^{-1} $\cdot$ min^{-1}) to reproduce fasting secretion rates in the rats with the three profiles (pulsatile, constant, and T2DM) used in dogs. In protocol 2, a second cohort of conscious rats were studied during a modified hyperinsulinemic euglycemic clamp during suppression of endogenous insulin secretion with insulin infused intraportally in the same three patterns as before but at a rate to mimic the postprandial state in the rats (70 pmol $\cdot$ kg^{-1} $\cdot$ min^{-1}). Third, to establish the efficacy of the same three patterns of insulin delivery on hepatic insulin signaling, anesthetized rats received the same three intraportal insulin infusions during suppression of endogenous insulin secretion for 10 min (protocol 3) or 30 min (protocol 4), with sequential liver biopsies obtained to measure insulin signaling, blood glucose being clamped. To overcome potential confounding effects of varying portal insulin concentrations on immediate insulin signaling, we repeated protocol 4 but after 120 min, increased the intraportal insulin infusion to a comparable constant high rate for 30 min before liver biopsy. Thus, the effect of antecedent pulsatile versus constant insulin delivery on insulin signaling in response to an identical increment in insulin concentration was evaluated (protocol 5). Finally, to extend the findings of the relatively short-term intraportal vein insulin infusions possible to a longer-term setting, we studied the HIP rat model of T2DM (n = 39) and age-matched Sprague Dawley controls (n = 36) at aged 2, 7, and 12 months. Intraportal vein insulin sampling and quantification of pulsatile insulin secretion undertaken as previously described (22) affirmed a progressive defect in pulsatile insulin secretion in the HIP rat. This model therefore permitted concurrent evaluation of hepatic glucokinase (*Gck*) gene expression, the major downstream insulin signaling target noted to be impaired with abnormal insulin delivery, in HIP and wild-type rats at aged 2, 7, and 12 months.

Glucose kinetics. Blood glucose enrichment was measured by gas chromatography mass spectrometry after extraction by ion exchange chromatography and derivatization to penta-acetate.

Immunoprecipitation and immunoblotting analysis. Tissue lysates were prepared from frozen liver samples and homogenized in standard lysis buffer A supplemented with the protease inhibitor cocktail (Roche, Mannheim, Germany). For immunoprecipitation of insulin receptor substrate (IRS)-1, IRS-2, and forkhead box class o (Foxo)1, 1 mg liver extracts were incubated with rabbit polyclonal antibodies against IRS-1, IRS-2, and Foxo1 overnight at 4°C. Protein A-agarose (Millipore, Temecula, CA) then was added and incubated for 2 h at 4°C. Immunocomplexes were resolved on 8% Bis-Tris ν-PAGE (Invitrogen). Phosphorylated or total protein was analyzed by immunoblotting with specific antibodies against IRS-1 (06–248), IRS-2, (06–506), p85 (06–195), and p-tyrosine (05–321) purchased from Millipore and antibodies specific for AKT (9272), AKT (Ser473) (9271), Foxo1 (2880), and Foxo1 (Ser256) (9461) purchased from Cell Signaling. Protein signals were detected with horseradish peroxidase–conjugated secondary antibodies (Invitrogen) using an enhanced chemiluminescence detection system. Images were analyzed and quantified using LabWorks Image Acquisition and Analysis software (UVP, Upland, CA).

Immunofluorescence. Liver tissue was fixed in 4% paraformaldehyde for 24 h at 4°C and embedded in paraffin. Sections (4 μm) were stained for Foxo1 (1:25 dilution) and glutamine synthase (1:100; BD Transduction Laboratories, San Jose, CA). Secondary antibodies labeled with Cy3 and fluorescein isothiocyanate were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and used at dilutions of 1:100 for 1-h incubation. Slides were viewed using a Leica DM6000 microscope (Leica Microsystems, Bannockburn, IL), and images were acquired using OpenLab software (Improvision, Lexington, MA).

Real-time PCR. Total RNA was isolated with the RNeasy kit (QIAGEN), and cDNA synthesis was performed using the SuperScript III First-Strand synthesis kit (Invitrogen) according to manufacturers' instructions. The real-time PCR was carried out with the LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche) using gene-specific primers following manufacturer's instructions.

Analytical procedures. Plasma glucose concentrations were measured using Beckman Glucose Analyzer. Insulin, C-peptide, and glucagon were measured in canine plasma samples by radioimmunoassay (Linco Research Inc., St. Louis, MO). Rat insulin was measured using an ELISA (ALPCO Diagnostics, Salem, NH). Plasma free fatty acid levels were measured using the colorimetric method (WAKO Chemicals, Richmond, VA).

Calculations. Insulin secretion rates were calculated as previously described in detail (10,22). The rates of hepatic glucose release during the last 60 min of the insulin replacement period were calculated by use of steady-state equations, the percent enrichment being confirmed to be at steady state (Supplementary Fig. 2).

Statistical analysis. Statistical analysis was performed using the ANOVA analysis, with Fisher post hoc when appropriate (Statsoft, Tulsa, OK). Significance was assigned at $P < 0.05$.

RESULTS

Effects of pulsatile insulin delivery on fasting glucose concentration and insulin sensitivity. To establish if the pattern of insulin concentration wave front presented to the liver in health is important for regulation of blood glucose and insulin sensitivity, first we studied dogs on three occasions with three different intraportal insulin infusion protocols. Concurrent portal vein sampling assured that the appropriate portal vein insulin concentration profiles were accomplished (Supplementary Fig. 1). With intraportal physiological pulsatile insulin delivery, the plasma glucose concentration decreased slightly as expected with continued fasting (85 ± 3 mg/dL after 300 min) (Fig. 1*C* and *D*). In contrast, when the same rate of insulin was infused in a constant manner, the plasma glucose concentration increased to the impaired fasting glucose concentration range (104 ± 3 mg/dL after 300 min, $P <$ 0.05 vs. pulsatile) (Fig. 1*C* and *D*). Moreover, when intraportal insulin delivery was infused to reproduce the attenuated pulses, and therefore decreased delivery rate present in T2DM (15), the plasma glucose concentration increased to values that would be adjudged as indicating diabetes (137 ± 11 mg/dL after 300 min, $P < 0.05$ vs. pulsatile) (Fig. 1*C* and *D*). Hepatic glucose release suppressed less despite higher blood glucose concentrations in both T2DM and constant insulin infusions compared with pulsatile insulin delivery (Fig. 1*E*). The insulin and glucagon concentrations in the systemic circulation were comparable in all three protocols throughout the study period (P = NS) (Fig. 1*F* and *G*). As expected, the endogenous plasma C-peptide concentrations decreased to the limits of detection during the somatostatin infusion during all three intraportal delivery protocols (P = NS) (Fig. 1*H*).

Next, we performed a study, comparable with that undertaken in dogs, in conscious rats with chronically implanted mesenteric vein catheters delivering the same pattern of three portal vein insulin infusions (Supplementary Fig. 3). As expected, the same pattern of blood glucose values in rats arose so that after 240 min of the pulsatile, constant, and T2DM pattern of intraportal insulin infusion, values were 88 ± 8 vs. 113 ± 6 vs. 160 ± 3 mg/dL, respectively ($P < 0.05$ vs. pulsatile) (Supplementary Fig. 3). These data imply that the pattern of insulin delivery into the portal vein influences insulin efficacy and signaling. To confirm this, we performed a modified hyperinsulinemic-euglycemic clamp in which insulin was infused via a mesenteric vein at a high physiological rate in the pulsatile, constant, or T2DM pattern while blood glucose values were clamped at euglycemia by a variable-rate glucose infusion (Supplementary Fig. 4). Insulin efficacy, assessed by the mean glucose infusion rate during this modified hyperinsulinemic-euglycemic clamp, was decreased with constant or T2DM insulin delivery compared with pulsatile (17 ± 2 and 11 ± 1 vs. 26 ± 2 mg/kg/min; $P < 0.05$ vs. pulsatile) (Supplementary Fig. 4).

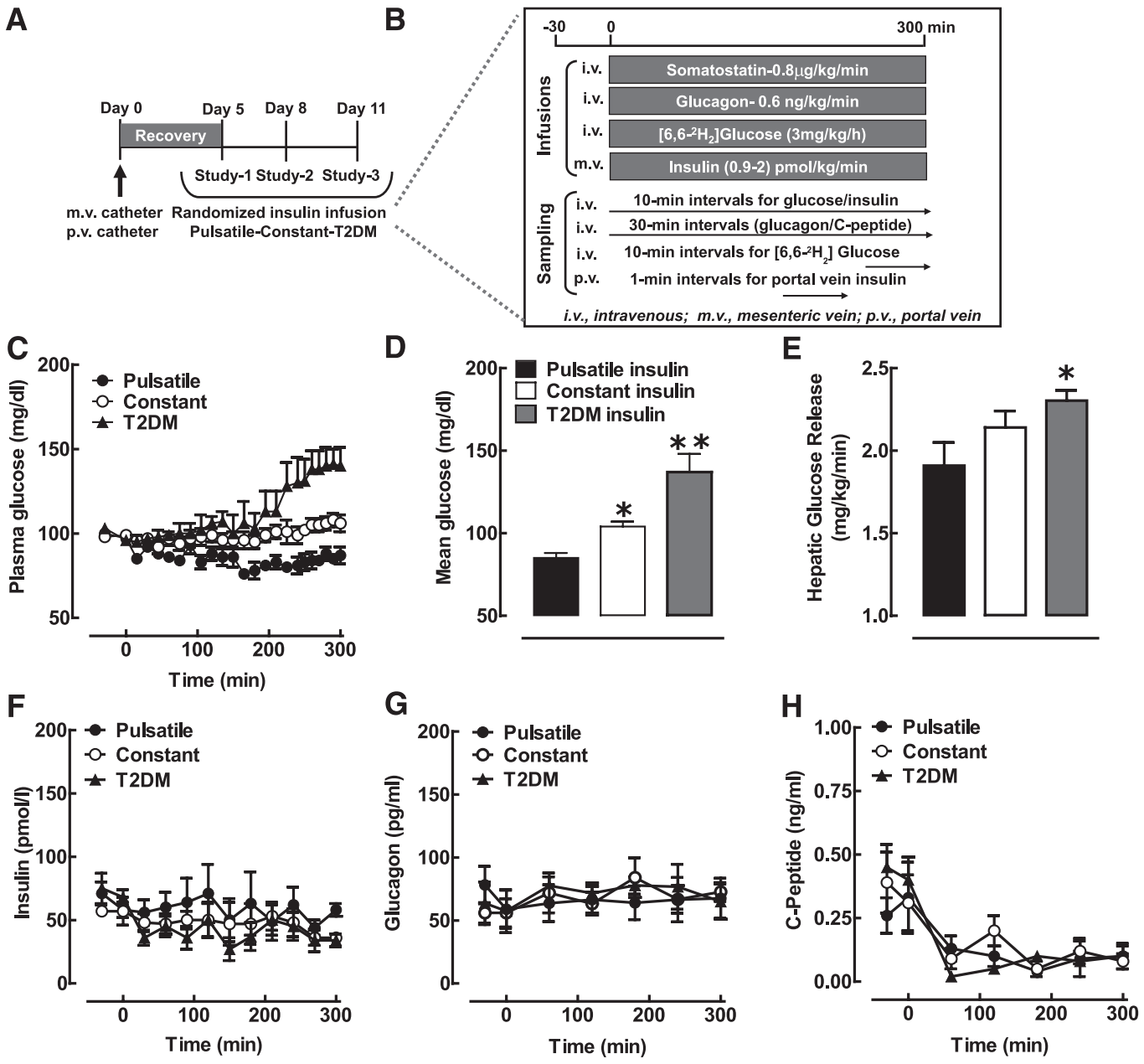

FIG. 1. Intraportal fasting insulin replacement protocol in dogs. ***A*****: All dogs had a portal vein sampling and mesenteric vein infusion catheter placed surgically under general anesthesia. After a 5-day recovery period, each dog was exposed in random order to** ***1*****) pulsatile portal vein insulin infusion,** ***2*****) constant portal vein insulin infusion, and** ***3*****) portal vein insulin infusion designed to reproduce that observed in patients with T2DM.** ***B*****: For each of these protocols, dogs were placed in a laboratory sling and somatostatin (0.8 μg/kg/min) and glucagon (0.65 ng/kg/min) were infused through the foreleg catheter throughout the study (0–300 min). A primed (3 mg/kg) continuous (3 mg/kg/h) infusion of [6, 6-$^2H_2$]glucose was given via foreleg catheter throughout the study to permit glucose turnover measurements. For the pulsatile protocol, insulin was infused into the mesenteric vein catheter with 70% of insulin delivered in pulses and 30% as a basal constant insulin infusion. For constant insulin infusion, the same total amount of insulin was delivered at a constant rate. For the T2DM protocol, insulin was delivered with 50% diminished pulses at the same frequency and with same basal insulin delivery.** ***C*****: Plasma glucose levels at baseline (−30 to 0 min) and during intraportal insulin replacement (0–300 min). Mean plasma glucose (*** ***D*****) and hepatic glucose production (*** ***E*****) during the steady-state intraportal insulin replacement period (260–300 min) after normal pulsatile (dark bar), constant (open bar), or T2DM (gray bar) intraportal insulin delivery in dogs. Systemic plasma levels of insulin (*** ***F*****), glucagon (*** ***G*****), and C-peptide (*** ***H*****) at baseline (−30 to 0 min) and during the intraportal insulin replacement study period (0–300 min). Data are mean ± SE. *P < 0.05 vs. pulsatile, **P < 0.05 vs. constant and pulsatile.**

Effects of pulsatile insulin delivery on hepatic insulin signaling. Having established that the intraportal pulsatile insulin concentration wave front present in health is more efficacious at regulating blood glucose than an equivalent constant infusion rate or the pattern of insulin delivery in T2DM, we next extended these findings to establish how these patterns of portal insulin delivery differ in their actions on the hepatic insulin signaling cascade. To address this, we established an anesthetized rat model with access to the liver for sequential liver biopsies. To evaluate the impact of the different patterns of portal vein insulin delivery on the proximal insulin signaling molecules, we undertook studies with four consecutive liver biopsy samples obtained during the first 10 min of portal vein insulin

infusion protocols (Figs. 2–4). To evaluate the impact of the different portal vein insulin profiles with an emphasis on the more distal insulin signaling molecules over a time frame consistent with putative varying actions of these molecules on glucose homeostasis (vide supra), we evaluated hepatic insulin signaling after 30 min of intraportal insulin delivery (Figs. 5 and 6).

For the 10-min sampling protocol (Fig. 2*A* and *B*), there were no differences in plasma glucose or free fatty acid concentrations between the three experimental protocols during the 10 min of the study (Fig. 2*D* and *E*). Intraportal delivery of insulin in a physiological pulsatile pattern accomplished acute and robust activation of both hepatic IRS-1 and IRS-2 signaling with increased phosphorylation of IRS-1– and IRS-2–associated phosphotyrosine (pY) and p85 subunit of phosphatidylinositol (PI) 3-kinase (Fig. 3). Activation of hepatic insulin signaling was delayed and impaired when insulin was delivered at the same rate in a constant manner with an ~50–80% reduction in both IRS-1 and IRS-2–associated pY and p85 subunit of PI 3-kinase ($P < 0.05$ vs. pulsatile) (Fig. 3). When the intraportal insulin delivery rate mimicked that in T2DM, IRS-1– and IRS-2–associated pY and p85 subunit of PI 3-kinase expression were impaired to a similar extent as that seen with the constant insulin infusion (Fig. 3). The delayed and impaired activation of IRS-1 and IRS-2 with constant or T2DM insulin pulses compared with physiological pulses was mirrored by a comparable defect in activation of the downstream insulin signaling effector molecules, such as AKT and Foxo1 (Fig. 4). Specifically, insulin-stimulated phosphorylation of AKT (at Ser473 residue) and Foxo1 (at Ser256 residue) was diminished ~50–80% ($P < 0.05$ vs. pulsatile) (Fig. 4) after constant or T2DM intraportal insulin delivery compared with typical pulsatile pattern.

To better recapitulate in vivo conditions, we next examined effects of more prolonged (30 min) exposure to intraportal insulin delivery (Figs. 5 and 6) on hepatic insulin signaling and gene expression, particularly focusing on

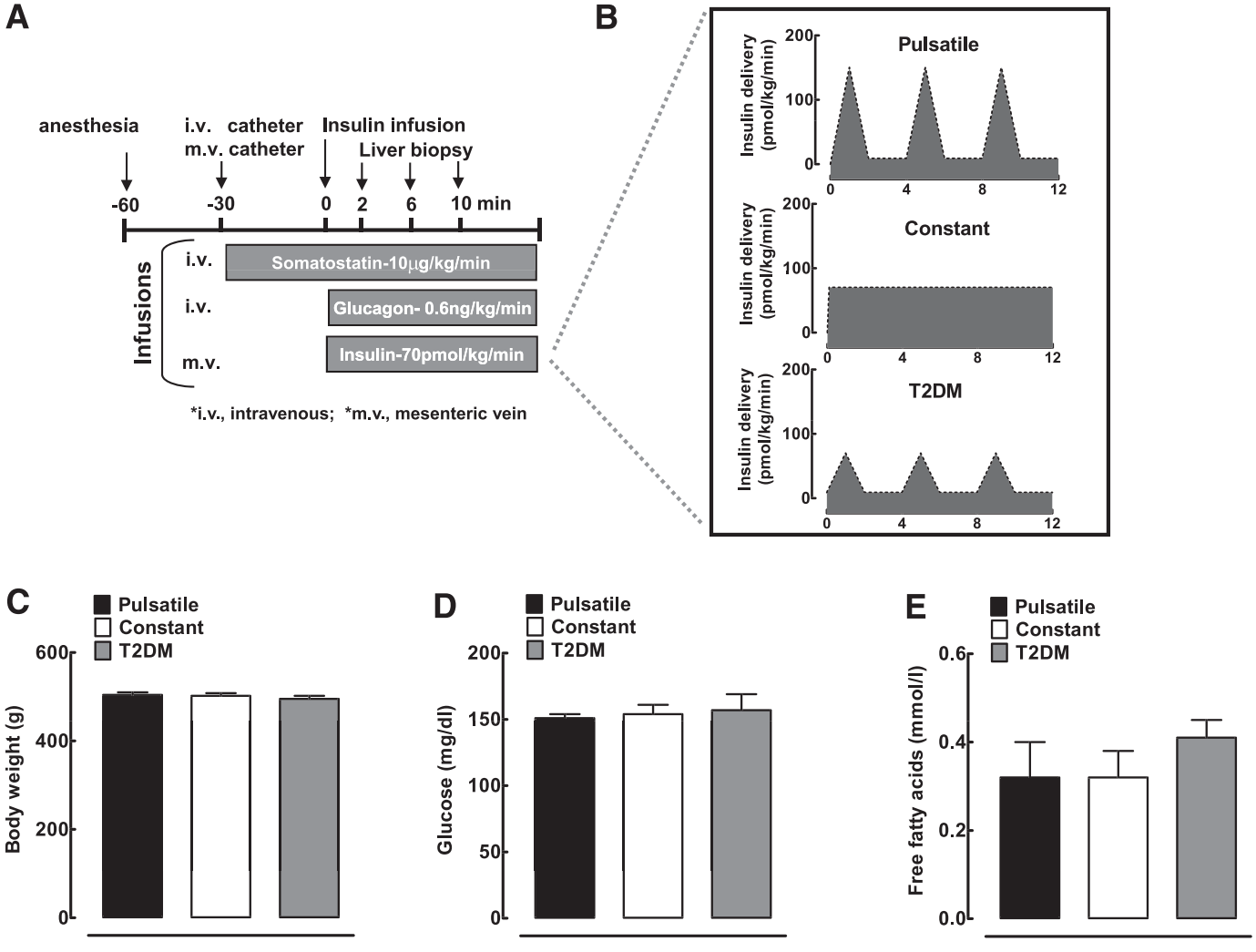

FIG. 2. Intraportal 10-min insulin delivery protocol in rats (rat protocol 3). *A* and *B*: Schematic representation of portal vein insulin infusion procedure in rats. Rats were anesthetized and indwelling catheters were placed into the mesenteric vein, jugular vein, and carotid artery. Rats received a constant intravenous infusion of somatostatin (10 μg/kg/min) for 30 min to inhibit endogenous insulin release. Insulin was infused intraportally at the rate of 70 pmol/kg/min either in a typical pulsatile pattern, in a same-rate constant insulin infusion, or as an insulin infusion selected to reproduce that observed in patients with evolving diabetes (~50% decrease in pulse mass). Sequential hepatic biopsies were obtained at 0, 2, 6, and 10 min after the start of insulin infusion. Body weight (*C*), plasma glucose (*D*), and free fatty acid (*E*) levels were not significantly different after the three modes of insulin delivery. Data are mean ± SE.

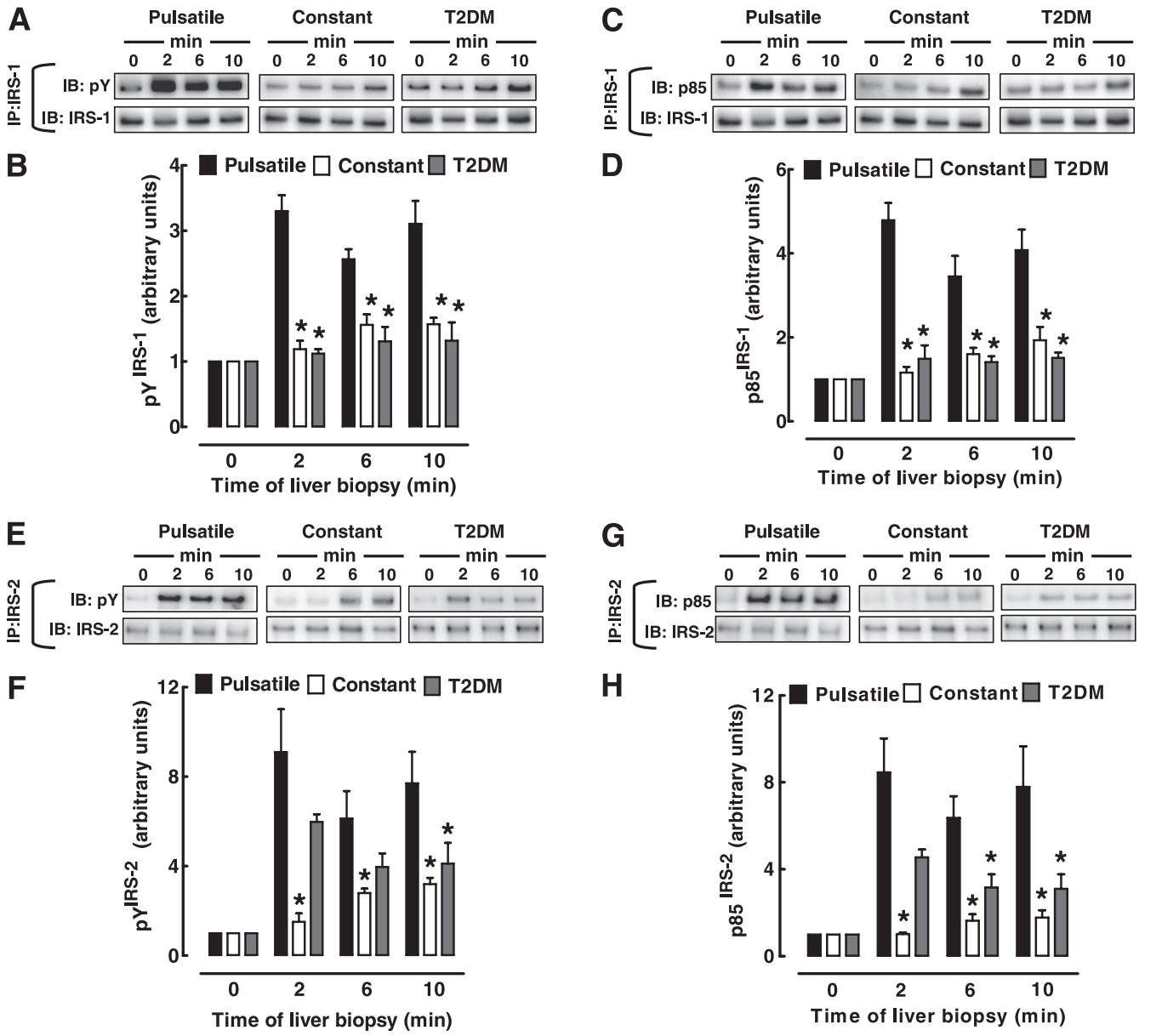

FIG. 3. IRS-1 and IRS-2 activation after pulsatile, constant, or T2DM intraportal 10-min insulin delivery protocol in rats. Immunoblot (IB) analysis of liver samples biopsied from rats exposed to 10 min of intraportal insulin delivered in either typical pulsatile fashion, same-rate constant infusion, or reduced pulses (T2DM). Hepatic biopsies were obtained at 0, 2, 6, and 10 min consecutively from the same liver after the start of portal vein insulin infusion. Activation of IRS-1 was examined by immunoprecipitation (IP) with an antibody against IRS-1, and the samples were then immunoblotted with antibodies against pY (*A* and *B*) or p85 subunit of PI 3-kinase (*C* and *D*). Activation of IRS-2 was examined by immunoprecipitation with an antibody against IRS-2, and the samples were then immunoblotted with antibodies against pY (*E* and *F*) or p85 subunit of PI 3-kinase (*G* and *H*). Data are mean ± SE. **P* < 0.05 vs. pulsatile.

activation of downstream insulin signaling targets AKT and Foxo as well as a key glucoregulatory gene, *Gck*. By design, the plasma glucose was clamped (Fig. 5*C*), and free fatty acid concentrations did not differ between groups during the 30-min observation period (Fig. 5*D*). The glucose infusion rate required to maintain euglycemia during the clamp was 50–70% decreased with the constant or T2DM intraportal insulin delivery ($P < 0.05$ vs. pulsatile) (Fig. 5*E*) compared with the physiological pulsatile infusion consistent with the studies in conscious dogs and rats. In addition, insulin-stimulated phosphorylation of AKT at Ser473 residue ($P < 0.05$ vs. pulsatile) and Foxo1 at Ser256 residue ($P = 0.09$ vs. pulsatile) were diminished ~50% after constant or T2DM intraportal insulin delivery compared with the normal pulsatile insulin delivery (Fig. 6*A* and *B*). Consistent with the impaired Foxo1 phosphorylation accomplished with either the constant or T2DM pattern of insulin delivery, Foxo1 nuclear exclusion was also decreased compared with that following the physiological pulsatile pattern of intraportal insulin delivery (Fig. 6*C*).

We used real-time PCR to establish whether changes in the pattern of hepatic insulin delivery influenced hepatic gene expression of genes that regulate glucose homeostasis. In particular, we focused on *Gck* since *1*) it plays a key role in regulating hepatic glucose fluxes (25), *2*) its

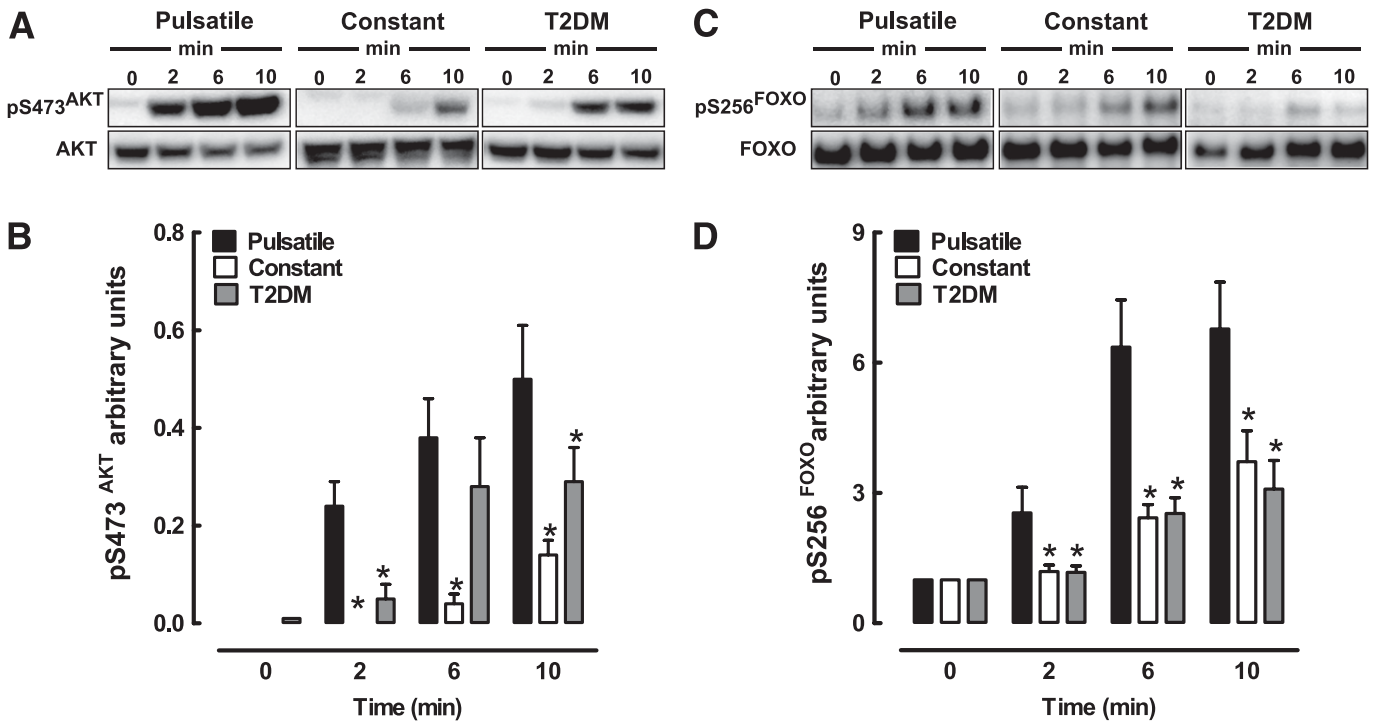

FIG. 4. AKT and FOXO activation after pulsatile, constant, or T2DM intraportal 10-min insulin delivery protocol in rats. Immunoblot analysis of biopsied liver samples from rats exposed to 10 min of portal vein insulin delivered in either typical pulsatile fashion, same-rate constant infusion, or reduced pulses (T2DM). Hepatic biopsies were obtained at 0, 2, 6, and 10 min consecutively from the same liver after the start of portal vein insulin infusion and immediately preserved using liquid nitrogen. Phosphorylation of AKT and FOXO was determined by immunoblotting with phosphospecific antibodies against AKT (phospho-Ser473) (*A* and *B*) and FOXO (phospho-Ser256) (*C* and *D*). Data are mean ± SE. *$P < 0.05$ vs. pulsatile.

activity is reduced in the liver in T2DM (26), and 3) its genetic inactivation in animals and humans results in hyperglycemia associated with dysregulation of hepatic glucose fluxes (27). As expected, *Gck* mRNA was increased (approximately twofold, $P < 0.05$ vs. baseline) after 30-min intraportal insulin delivery in a typical pulsatile pattern (Fig. 6*D*). In contrast, *Gck* mRNA did not increase after insulin delivery in either a constant or T2DM infusion (Fig. 6*D*).

Next, we examined whether antecedent intraportal pulsatile versus constant insulin delivery affects hepatic insulin signaling in response to an identical subsequent increment in insulin concentration. This approach thus allowed us to avoid potential confounding effects of minute-by-minute insulin concentration changes on hepatic insulin signaling. This approach revealed that antecedent pulsatile (vs. constant) insulin delivery resulted in increased *Gck* mRNA and AKT activation in response to an identical increment in insulin delivery (Fig. 7).

Finally, we studied the HIP rat model of T2DM to extend the findings over short periods possible during catheterization studies (protocols 1–5) to longer periods relevant in human diabetes. The HIP rat model of T2DM develops a progressive defect in insulin secretion and β-cell mass as a result of misfolding and accumulation of toxic oligomers of human islet amyloid polypeptide developing an islet phenotype comparable in many respects with that in humans with T2DM (6). We now report that defective insulin secretion in the HIP rat mirrors that in humans with T2DM as a result of a progressive defect in insulin secretory pulse mass (Fig. 8). Moreover, there was a progressive defect in hepatic *Gck* mRNA in the HIP rat that mirrors the progressive decline in pulsatile insulin secretion and, thus, lends further support for the importance of pulsatile insulin secretion in regulation of hepatic *Gck* gene expression.

DISCUSSION

Impaired fasting glucose and/or impaired glucose tolerance, often precursors of T2DM, are characterized by β-cell dysfunction and hepatic insulin resistance (4,5,28). Insulin resistance is also a prominent feature of early T1DM (3) but is reversed to an extent that β-cell function is restored in the early treatment phase (3). This raises the question, does β-cell dysfunction cause hepatic insulin resistance and thereby set up an adverse positive feedback cycle that precipitates loss of glycemic control and diabetes onset? Since insulin is predominately secreted in a pulsatile manner (10,11) and dysregulation of these pulses is an early defect in T1DM and T2DM (29,30), we tested the hypothesis that the pulsatile insulin concentration wave front presented to the liver is important for appropriate insulin signaling and action.

Prior studies report that pulsatile insulin delivery into the systemic circulation is more efficacious than constant insulin infusion (17–19). However, given the volume of distribution of insulin in the systemic circulation, it is not possible to reproduce the insulin concentration wave front presented to hepatocytes in health through this mode of delivery (10). One study examined delivery of insulin into the portal vein in pulses or a constant manner during hyperglycemia (200 mg/dL) and reports no difference in hepatic glucose uptake (20). However, in that study, insulin was delivered at a rate designed to fully suppress hepatic glucose release so that no effect of pulsatile insulin delivery on hepatic glucose release was possible,

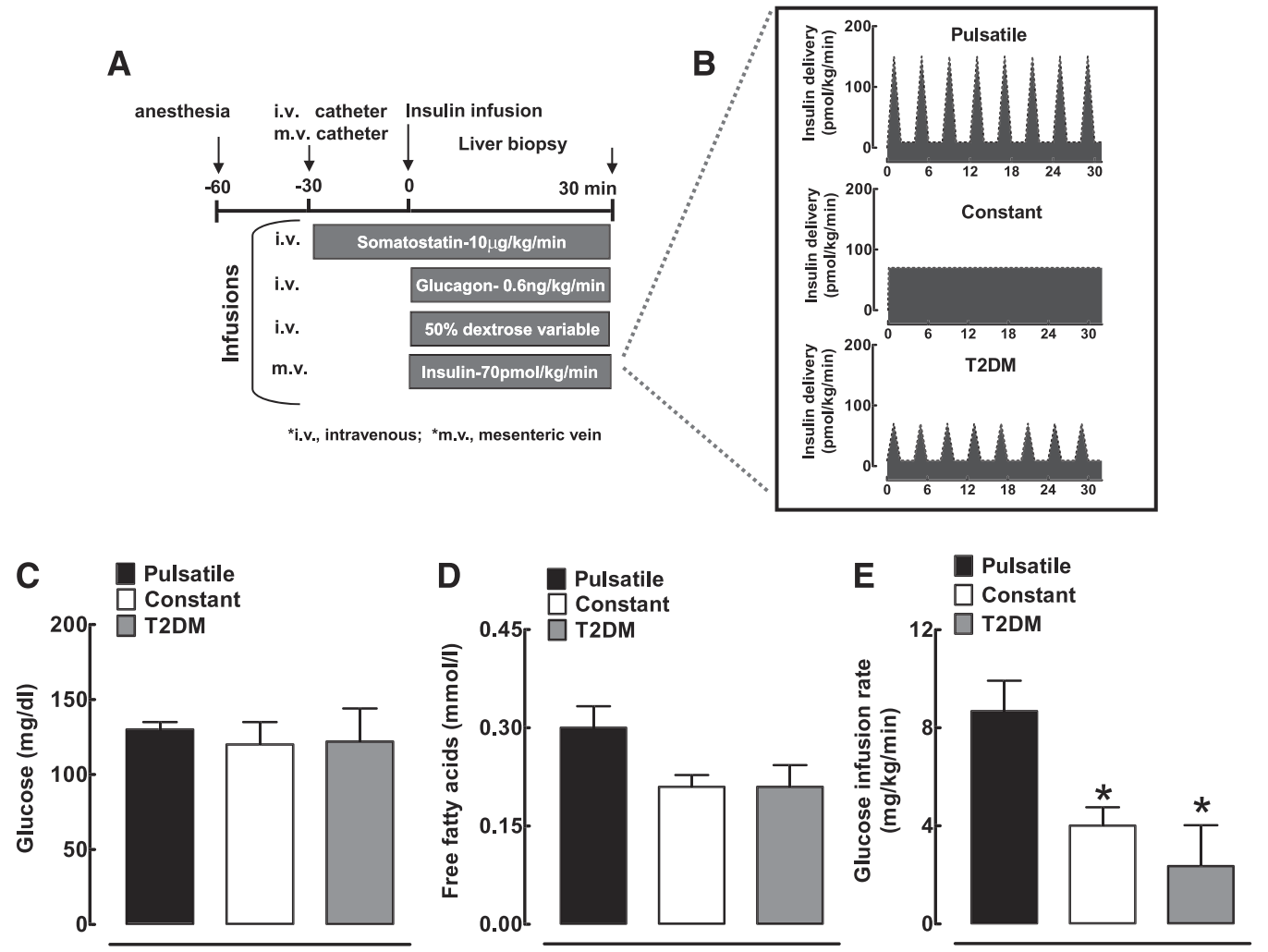

FIG. 5. Intraportal 30-min insulin delivery protocol in rats (rat protocol 4). *A*: Rats were anesthetized and indwelling catheters were placed into the mesenteric vein, jugular vein, and carotid artery. *B*: Rats received a constant infusion of somatostatin (10 μg/kg/min) for 30 min to inhibit endogenous insulin release. Insulin was infused directly into the portal vein at the rate of 70 pmol/kg/min either in a typical pulsatile pattern, in a same-rate constant insulin infusion, or as an insulin infusion selected to reproduce that observed in patients with evolving diabetes (~50% decrease in pulse mass). A variable infusion of 50% dextrose was given intravenously to match systemic plasma glucose concentrations among the three groups. Sequential hepatic biopsies were obtained at 0 and 30 min after the start of insulin infusion protocols. Plasma glucose (*C*), free fatty acid (*D*), and mean glucose infusion rates (*E*) required to match plasma glucose levels among the three infusion protocols. Data are mean ± SE. **P* < 0.05 vs. pulsatile.

and insulin signaling was not evaluated. Moreover the insulin pulse frequency delivered in those studies (12-min pulse interval) differed from that used here (6-min pulse interval), the latter reflecting the insulin pulse frequency established in vivo using validated methods for pulse detection measured directly in the portal vein (10,31).

The findings of the current study support the hypothesis that the pulsatile mode of insulin secretion delivered into the portal vein is more efficacious than a comparable rate of constant intraportal vein insulin delivery. Furthermore, intraportal infusion of the T2DM pattern of insulin pulses in the same dogs resulted in a blood glucose concentration profile classified diabetes (Fig. 1), despite insulin concentrations in the systemic circulation comparable with the control insulin infusion.

In the current study, having established that intraportal delivery of insulin in physiological insulin pulses is important for insulin action, we then examined the mechanism subserving this by studying activation of insulin signaling pathways in the rat liver exposed to the same three patterns of insulin delivery. Activation of both the IRS-1 and IRS-2 limbs of the insulin signaling pathways was delayed and attenuated with either the constant or T2DM pattern of insulin pulses compared with control pulses. Moreover, the impaired activation of Foxo1 by the constant or T2DM pattern of insulin delivery is consistent with the impaired insulin action by those modes of insulin delivery (32,33). Also, given the important role of Foxo1 in regulation of hepatic lipid metabolism (34,35), the association between insulin resistance and increased risk of cardiovascular disease in both T1DM and T2DM may be mediated at least in part by impaired pulsatile insulin delivery to the liver (36,37).

Impaired insulin-mediated activation of Foxo1, as reported in response to defective pulsatile insulin delivery here, would be predicted to lead to impaired expression of *Gck* and favor gluconeogenesis over glycolysis with inappropriately increased hepatic glucose release and hyperglycemia (38). This is the pattern of abnormal hepatic glucose metabolism noted in humans with both T1DM and T2DM

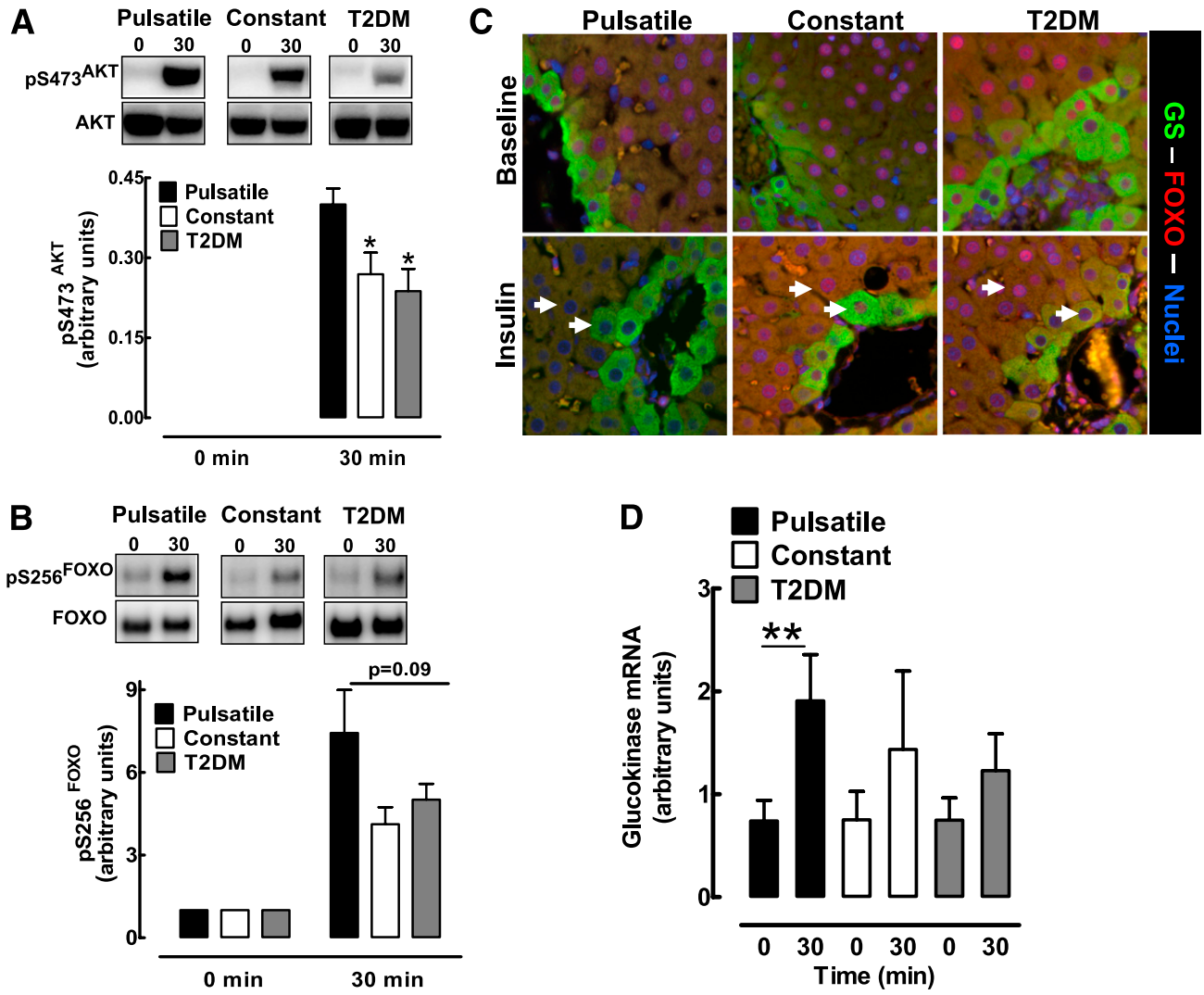

FIG. 6. AKT, FOXO, and *Gck* mRNA activation after pulsatile, constant, or T2DM intraportal 30-min insulin delivery protocol in rats. Immunoblot analysis of liver samples from rats exposed to 30 min of portal vein insulin delivery in either typical pulsatile fashion, same-rate constant infusion, or reduced pulses (T2DM). Hepatic biopsies were obtained at 0 and 30 min after the start of insulin infusion and immediately preserved using liquid nitrogen. Phosphorylation of AKT and FOXO was determined by immunoblotting with phosphospecific antibodies against AKT (phospho-Ser473) (*A*) and FOXO (phospho-Ser256) (*B*). *C*: FOXO nuclei exclusion after insulin delivery was examined by immunofluorescent staining for FOXO (red), perivenous hepatocyte marker glutamine synthetase (GS) (green), and nuclear marker DAPI (blue). *D*: *Gck* mRNA expression was determined by real-time PCR in liver samples from rats exposed to 30 min of portal vein insulin delivered in either typical pulsatile fashion, same-rate constant infusion, or reduced pulses (T2DM). Data are mean ± SE. *$P < 0.05$ vs. pulsatile, **$P < 0.05$ vs. min-0. (A high-quality digital representation of this figure is available in the online issue.)

(2,39–41). Moreover, the same pattern of defective hepatic glucose metabolism is present in patients with diabetes due to mutant *Gck* (MODY2) (42). It is therefore noteworthy that we find defective hepatic *Gck* expression observed with either the T2DM or constant pattern of insulin delivery reported here, and, indeed, this is consistent with reported decreased hepatic *Gck* activity in the liver of individuals with T2DM (26). It is of note that in the present studies, the glucagon concentrations were deliberately comparable in all three insulin infusion protocols. Since there is relative hyperglucagonemia in both T1DM and T2DM, the decreased *Gck* expression consequent upon abnormal portal vein insulin delivery would potentially be further diminished (43). We extended the intraportal insulin infusion studies by examining hepatic gene expression in the HIP rat model of T2DM. First, we established that the progressive defect of insulin secretion in this model mirrors that in T2DM in humans, being characterized by a defect in insulin pulse mass with no change in pulse frequency (15,16). This defect in insulin pulse mass was accompanied by impaired mRNA expression of hepatic *Gck*, consistent with the short-term intraportal vein insulin infusion studies reported here as well as defective hepatic *Gck* expression in humans with T2DM (26).

Hepatic insulin clearance decreases if intraportal vein insulin secretion declines because of decreased insulin secretory burst mass and pulse amplitude (13,44,45), likely explaining the decreased hepatic insulin clearance of endogenously secreted insulin in T2DM (46). Hepatic insulin clearance is an insulin receptor–mediated process, and after the insulin receptor and insulin ligand interact, the receptor internalizes as downstream signaling is effected before the receptor is returned to the cell surface ~4 min later (47,48). The timing of the insulin receptor to complete this itinerary thus is perfectly suited to entrain to the episodic delivery of insulin via the sinusoids directly to hepatocytes (48).

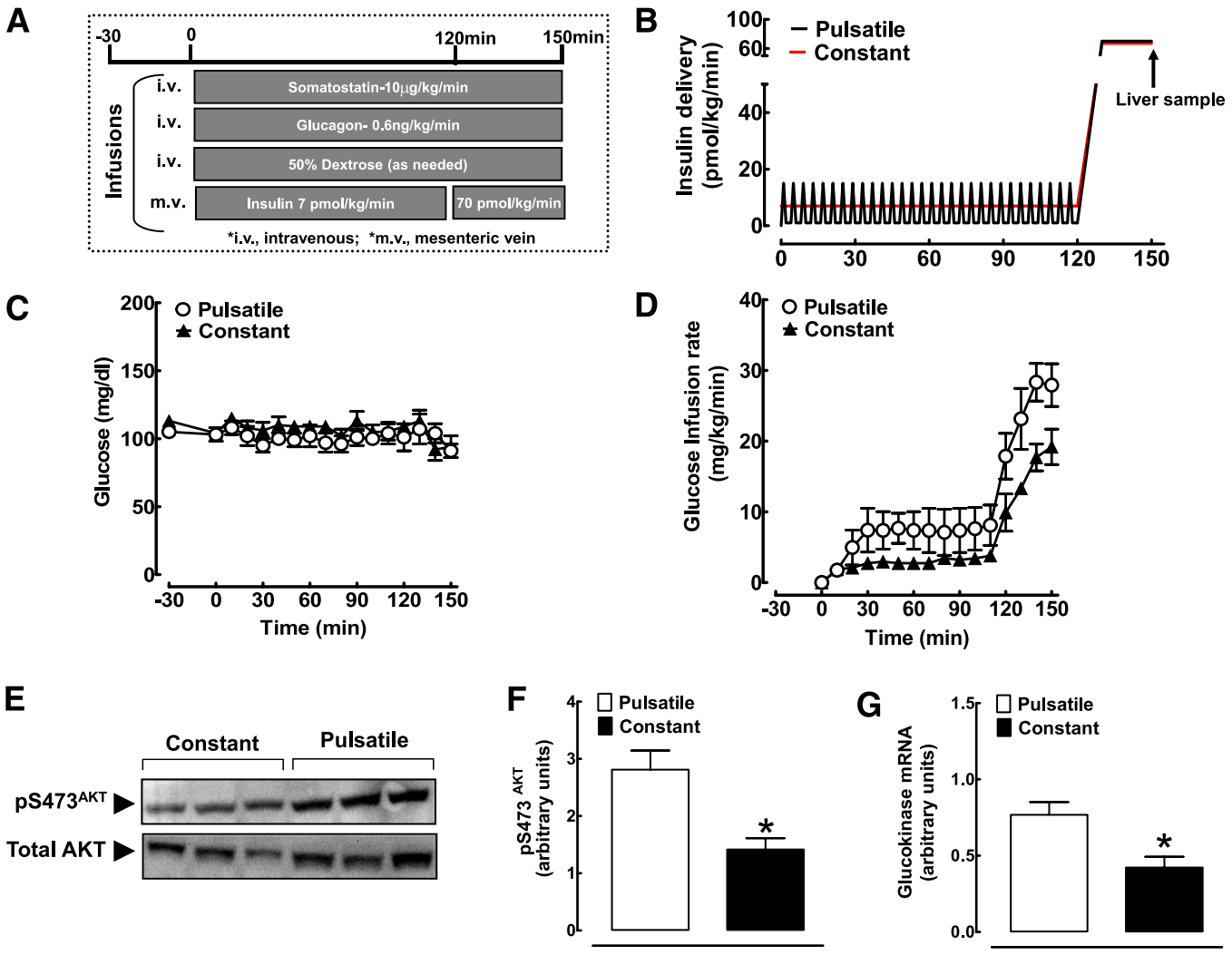

FIG. 7. Effects of antecedent pulsatile vs. constant insulin delivery on hepatic insulin signaling and gene expression in rats (rat protocol 5). *A*: Antecedent portal vein insulin infusion protocol. In short, all rats had a mesenteric and jugular vein infusion catheter and carotid artery sampling catheter placed surgically under anesthesia; after 6-day recovery, two random cohorts of rats (n = 4 per group) underwent a 150-min study protocol. During somatostatin inhibition of endogenous insulin secretion, one cohort of rats (pulsatile) received 120-min insulin infusion into the mesenteric vein at a basal physiological rate (7 pmol/kg/min) in 5-min pulses to recapitulate fasting insulin secretion. Another cohort of rats (constant) received the same rate (7 pmol/kg/min) 120-min insulin infusion into the mesenteric vein, but as a constant infusion. After 120-min portal vein antecedent insulin infusion, all rats received an identical 30-min bolus of insulin (70 pmol/kg/min) designed to recapitulate the postprandial rise in insulin secretion. After the 30-min bolus insulin infusion, all rats were euthanized and livers quickly removed for subsequent analysis of protein and gene expression. Dextrose (50%) was infused at variable rates throughout the 150-min study protocol to clamp plasma glucose concentrations at basal levels. *B*: Hepatic portal vein insulin infusion rates in rats receiving 120-min pulsatile vs. constant antecedent insulin delivery. Plasma glucose levels (*C*) and mean glucose infusion rates (*D*) during the 150-min study protocol in rats receiving 120-min antecedent pulsatile or constant insulin delivery into the hepatic portal vein. *E* and *F*: Immunoblot analysis of liver samples from rats exposed to either antecedent pulsatile or constant portal vein insulin delivery. Hepatic biopsies were obtained at the end of the 150-min study protocol and immediately preserved using liquid nitrogen. Phosphorylation of AKT was determined by immunoblotting with phosphospecific antibodies against AKT (phospho-Ser473). *G*: *Gck* mRNA expression was determined by real-time PCR. Data are mean ± SE. **P* < 0.05 vs. pulsatile. (A high-quality color representation of this figure is available in the online issue.)

Moreover, the optimal insulin insulin-receptor binding affinity occurs at a concentration reproduced by the insulin concentration wave front delivered by the insulin pulses (48,49). In addition, intermittent insulin delivery may permit more efficient transmission of insulin signaling by avoiding negative feedback. Thus, the action of downstream insulin signaling targets to inhibit proximal insulin signaling effecter molecules might decay before the arrival of the next signal (50).

Circulating free fatty acids have also been reported to influence the actions of insulin in regulating hepatic glucose release (51). In the present studies, we did not observe any differences in circulating free fatty acids with the three intraportal insulin infusion protocols, presumably because systemic insulin concentrations were comparable (21). The beneficial actions of pulsatile insulin delivery on hepatic insulin signaling reported here therefore do not appear to be mediated by changes in free fatty acid concentrations.

In conclusion, hepatic insulin signaling is delayed and impaired when insulin is delivered in a nonpulsatile manner or that which reproduces defective insulin secretion in T2DM. The relatively early development of hepatic insulin resistance in T1DM and T2DM may thus in part be a consequence of β-cell dysfunction and loss of the optimal pattern of pulsatile insulin delivery. The

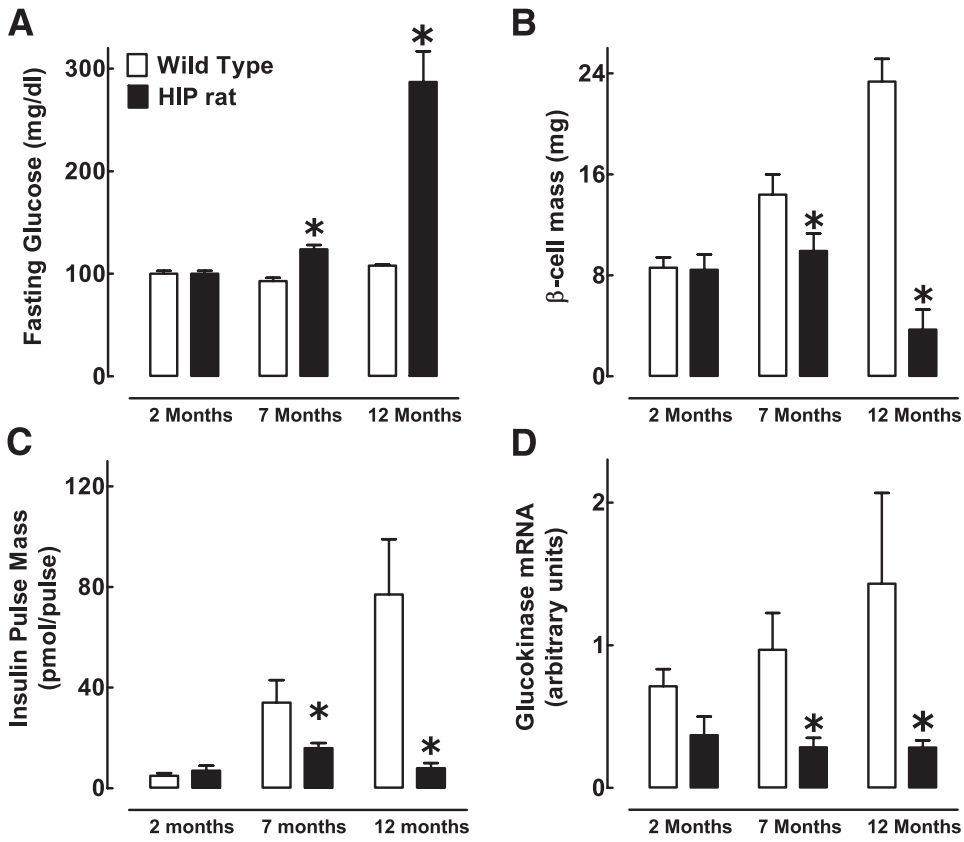

FIG. 8. Progressive loss of insulin secretory pulse mass in HIP rats is associated with the reduction in *Gck* mRNA expression (rat protocol 6). The mean fasting plasma glucose (*A*), β-cell mass (*B*), insulin secretory pulse mass (*C*), and hepatic *Gck* mRNA expression (*D*) in wild-type and HIP rats aged 2, 7, and 12 months. Data are mean ± SEM. **P* < 0.05 for HIP vs. wild type.

resulting increased β-cell workload on failing β-cells in evolving T1DM or T2DM likely hastens β-cell failure, collectively leading to diabetes onset.

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A.V.M. assisted with design of the studies, performed studies, and assisted with interpretation of the studies and preparation of the manuscript. D.L., T.G., and D.K. assisted with performing studies. C.D.M. assisted with the calculations and interpretation of studies. C.C. assisted with the computations and interpretation of the studies. M.F.W. assisted with the design and interpretation of the studies. K.D.C. assisted with the design and planning of the studies. E.V. assisted with the execution and interpretation of the studies. S.F. assisted with the execution of the studies. P.C.B. contributed to the design and interpretation of the studies and preparation of the manuscript. A.V.M. is the guarantor of this work and, as such, had full access to all of 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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