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Novel PEGylated Basal Insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism

The impact of the novel basal insulin LY2605541 (LY) on hepatic and nonhepatic glucose uptake (non-HGU) was evaluated. Conscious dogs underwent euglycemic clamps with tracer and hepatic balance measurements. Clamp period infusions were peripheral venous regular insulin (0.1 nmol $\cdot$ kg^{-1} $\cdot$ h^{-1} [control], $n = 6$) or LY (bolus [nmol/kg], continuous [nmol $\cdot$ kg^{-1} $\cdot$ h^{-1}]: 0.5, 0.5 [$n = 6$]; 0.375, 0.375 [$n = 5$]; 0.25, 0.25 [$n = 4$]), somatostatin, and glucose, as well as intraportal glucagon (basal). During the clamp, the dogs switched from net hepatic glucose output to uptake (rates reached 2.1 ± 1.2, 0.9 ± 2.1, 8.6 ± 2.3, and 6.0 ± 1.1 μmol $\cdot$ kg^{-1} $\cdot$ min^{-1} within 5 h in control, LY$_{0.25}$, LY$_{0.375}$, and LY$_{0.5}$, respectively). Non-HGU in LY increased less than in control; the ratio of change from basal in non-HGU to change in net hepatic glucose balance, calculated when glucose infusion rates (GIRs) were $\sim$20 μmol $\cdot$ kg^{-1} $\cdot$ min^{-1} in all groups, was higher in control (1.17 ± 0.38) versus LY$_{0.25}$ (0.39 ± 0.33), LY$_{0.375}$ (-0.01 ± 0.13), and LY$_{0.5}$ (-0.09 ± 0.07). Likewise, the change from baseline in glucose R_d-to-R_a ratio was greatest in control (1.4 ± 0.3 vs. 0.6 ± 0.4, 0.5 ± 0.2, and 0.6 ± 0.2 in LY$_{0.25}$, LY$_{0.375}$, and LY$_{0.5}$, respectively). In contrast to exogenously administered human insulin, LY demonstrated preferential hepatic effects, similar to endogenously secreted insulin. Therefore, the analog might reduce complications associated with current insulin therapy.

Marked improvements have been made in insulin analogs for basal-bolus therapy over the last few years. Insulins detemir and glargine reduce the risk of hypoglycemia compared with the older NPH insulin (1) and come closer to the ideal of creating a peakless basal insulin (2). However, neither detemir nor glargine maintains 24-h basal insulinemia for all individuals on all days (3), and thus improved analogs are needed. Insulin degludec, approved for use in Europe, Japan, and Mexico, is the first of the next-generation analogs to reach the market. The $T_{1/2}$ of insulin degludec is approximately two times that of glargine (4), with the prolonged activity being achieved by the formation of soluble multihexamers upon subcutaneous injection, followed by gradual release of insulin monomers from the aggregate (5). Compared with glargine in treat-to-target trials, insulin degludec has brought about similar reduction in HbA$_{1c}$ at lower dosages and with lower rates of nocturnal hypoglycemia (6,7). Another new basal insulin, LY2605541 (LY), is currently in phase 3 trials.

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LY is insulin lispro with a 20-kDa polyethylene glycol moiety covalently attached to lysine B28 via a urethane bond (8,9). Its large hydrodynamic (i.e., functional) size (10) may contribute to slowing absorption and reducing clearance, resulting in prolonged duration of action. In phase I trials, the half-life of LY was between 1 and 3 days and reached steady-state concentrations within 7–10 days with ~8- to 13-fold increase in exposure at steady-state. After a single dose, the requirement for glucose infusion was at least 36 h, indicating prolonged pharmacodynamic action. At steady-state, nearly peak-less glucose infusion profiles were observed due to low peak-to-trough pharmacokinetic fluctuation (11,12). In two phase II trials in patients with type 1 and type 2 diabetes, LY was associated with a lower rate of nocturnal hypoglycemia and glucose variability compared with insulin glargine. At the same time, it resulted in similar glycemic control in the type 2 diabetic patients but better glycemic control in the type 1 diabetic patients. In the type 1 diabetic patients, there was a slightly higher rate of total hypoglycemia but a lower prandial insulin requirement (13,14). In addition, these patients, who were previously treated with other basal insulins, showed modest weight loss when switched to LY compared with modest weight gain with insulin glargine. They also exhibited modestly higher triglycerides compared with those on insulin glargine (13,14). These effects prompted an examination of the effect of LY on specific tissues, including the liver, kidney, and skeletal muscle. The chronically catheterized conscious dog was chosen as the model because it permits cannulation of the vessels required for assessing glucose balance across these organs/tissues and because of the similarity in insulin's regulation of glucose metabolism in the human and the dog.

RESEARCH DESIGN AND METHODS

Animals and Surgical Procedures

The protocol was approved by the Vanderbilt University Institutional Animal Care and Use Committee, and the animals were housed and cared for according to Association for Assessment and Accreditation of Laboratory Animal Care International guidelines. Approximately 16 days prior to study, each dog underwent surgical preparation under general anesthesia. In the initial studies (part A; $n = 12$), sampling catheters were placed in a femoral artery, the hepatic portal vein, the left common hepatic vein, a renal vein, and an iliac vein; infusion catheters were inserted in a jejunal and a splenic vein; and ultrasonic flow probes (Transonic Systems, Ithaca, NY) were placed around the hepatic artery, the portal vein, a renal artery, and an external iliac artery as previously described (15–18). In follow-up experiments (part B), the renal and iliac cannulas and flow probes were not placed, but all other aspects of the preparation were as described above. Criteria for use in a study were as previously described (17).

Experimental Protocol

On the morning of study, the proximal ends of the flow probes and catheters were exteriorized under local anesthesia. A primed, continuous infusion of [3-^{3}H]glucose was initiated via a peripheral vein, and the dog was allowed to rest quietly in a Pavlov harness throughout the period of tracer equilibration (90 min). This was followed by 30 min of baseline sampling (90–120 min) and a euglycemic clamp period lasting 5 h (from 120 to 420 min) in part A. At 120 min, infusions of somatostatin (Bachem, Torrance, CA) and insulin were begun via a peripheral vein, while glucagon was administered intraportally at a basal rate (0.5 ng · kg^{-1} · min^{-1}). The dogs in part A were randomly assigned to receive LY initiated with a 0.5 nmol/kg priming dose followed by continuous infusion at 0.5 nmol · kg^{-1} · h^{-1} (LY$_{0.5}$ group, $n = 6$) or regular human insulin (control group, $n = 6$) at 0.11 nmol · kg^{-1} · h^{-1} with no priming dose. The analog is designed for subcutaneous injection, but it was infused via peripheral vein in these experiments in order to come to near–steady-state in a reasonable period of time and to mimic the route by which subcutaneously injected insulin arrives at the liver and other insulin-sensitive tissues. There is no known modification of the molecule during the absorption from the subcutaneous tissues, and after absorption it circulates in the same manner as endogenous insulin.

The insulin infusion rates used (determined in pilot studies; data not shown) in the two groups required equivalent glucose infusion rates (GIRs) to maintain euglycemia over the time course of the experiments. Glucose (50%; Hospira, Lake Forest, IL) was infused via peripheral vein as needed to maintain euglycemia. All sampling catheters functioned with the exception of a renal catheter in one LY$_{0.5}$ animal and a hindlimb catheter in one control dog. At the end of each experiment, the dog was anesthetized, and hepatic biopsies were freeze clamped in situ as previously described (19). Subsequently, the dog was killed and the hypothalamus was removed and freeze clamped (20). Tissues were stored at $-80°$C to await further analysis.

Part B studies were identical to those in part A, except that the euglycemic clamp lasted 7 h (from 120 to 540 min), and the dogs were randomized to either the LY$_{0.375}$ ($n = 5$; 0.375 nmol/kg prime, 0.375 nmol · kg^{-1} · h^{-1} infusion) or LY$_{0.25}$ ($n = 4$; 0.25 nmol/kg prime, 0.25 nmol · kg^{-1} · h^{-1} infusion) groups. The purpose of part B was to determine whether the hepato-preferential effects of LY, observed in part A, would be evident at different dosages of the analog. Additionally, the longer clamp period was instituted to determine whether lower LY infusion rates sustained the hepato-preferential effects.

Sample Analyses

Plasma glucose, [^{3}H]glucose, glucagon, canine and human insulin, and nonesterified fatty acid (NEFA) levels and blood lactate and glycerol concentrations were measured

using standard methods as previously described (19,21). Plasma triglycerides were assayed spectrophotometrically (TR0100; Sigma-Aldrich, St. Louis, MO).

Serum concentrations of LY were assayed using a dual-antibody ELISA specific for LY (Charles River Laboratories, Senneville, QC, Canada). The quantitation range was 20–500 pmol/L, with the standard curve range 15–1,000 pmol/L. Samples with concentrations higher than 500 pmol/L were diluted so that the resulting concentration was within the quantitation range. Standard curve and quality control samples were analyzed with each set of study samples and passed acceptance criteria (within ±20% except at lower limit of quantitation: within ±25%).

Western blotting was carried out on hepatic and hypothalamic tissue from control and $LY_{0.5}$ dogs; all analytic procedures have previously been described (22). Antibodies against total and phosphorylated Akt (Ser^{473}), signal transducer and activator of transcription 3 (STAT3) (Tyr^{705}), and glycogen synthase kinase-3β (GSK3β) (Ser^9) were purchased from Cell Signaling (Danvers, MA). Protein bands were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

Calculations

Organ balance calculations and estimation of hepatic sinusoidal hormone concentrations and net hepatic carbon retention were carried out as previously described in detail (15–18). Tracer-determined glucose turnover was calculated with the circulatory model of Mari et al. (23). Unidirectional hepatic glucose uptake was calculated endogenous glucose R_a minus net hepatic glucose balance.

Two-way repeated-measures ANOVA (group × time) was used for statistical analysis of time course data, with post hoc analysis by Tukey test (Sigmaplot 11; Systat Software, San Jose, CA). Since part A was 2 h shorter than part B, statistical comparisons do not include the data from the last 2 h of part B. Unpaired t tests were used for comparison of tissue analytes. A P value <0.05 was considered statistically significant.

RESULTS

Hormone Concentrations and Hepatic Blood Flow

Portal vein blood flow declined 15–25% in response to somatostatin infusion ($P < 0.05$ for Δ from baseline in all groups), while there was a slight (albeit nonsignificant) 0–10% rise in hepatic artery blood flow (Table 1).

Arterial plasma C-peptide concentrations declined to near the limits of detection in all groups during the clamp period, demonstrating that insulin secretion was fully suppressed (data not shown). Arterial and hepatic sinusoidal glucagon concentrations remained near basal throughout the clamp period and did not differ significantly among groups ($P = 0.22$) (Table 1).

Plasma insulin concentrations during the baseline period were similar in all groups (data shown for control group only [Fig. 1A]). In the control group, the arterial

TABLE 1—Hepatic blood flow and plasma glucagon concentrations

	Baseline period	Clamp period
Hepatic artery blood flow (mL · kg^{-1} · min^{-1})		
Control	6.9 ± 1.2	7.3 ± 1.1
$LY_{0.5}$	5.7 ± 0.7	6.3 ± 0.7
$LY_{0.375}$	6.8 ± 0.5	7.4 ± 0.7
$LY_{0.25}$	5.3 ± 0.7	5.2 ± 0.9
Portal vein blood flow (mL · kg^{-1} · min^{-1})		
Control	25.8 ± 1.5	19.5 ± 1.5
$LY_{0.5}$	28.0 ± 2.3	21.7 ± 1.5
$LY_{0.375}$	25.0 ± 1.8	21.2 ± 1.7
$LY_{0.25}$	22.2 ± 2.2	17.3 ± 1.3
Arterial plasma glucagon (ng/L)		
Control	50 ± 3	43 ± 2
$LY_{0.5}$	50 ± 6	45 ± 4
$LY_{0.375}$	50 ± 3	45 ± 5
$LY_{0.25}$	62 ± 8	55 ± 8
Hepatic sinusoidal plasma glucagon (ng/L)		
Control	58 ± 3	52 ± 2
$LY_{0.5}$	54 ± 6	57 ± 7
$LY_{0.375}$	56 ± 6	55 ± 5
$LY_{0.25}$	74 ± 4	67 ± 6

Data are mean ± SEM. n = 6/group for control and $LY_{0.5}$; n = 5 for $LY_{0.375}$, and n = 4 for $LY_{0.25}$. Basal period values are the mean of two sampling times (90 and 120 min); clamp period values are the mean of the sampling points between 120 and 420 min (for control and $LY_{0.5}$) and between 120 and 540 min (for $LY_{0.375}$ and $LY_{0.25}$). There were no significant differences among groups.

concentrations during the clamp period were twofold basal levels, but peripheral venous insulin delivery resulted in a fall in the portal vein concentrations to ~50% of basal. Consequently, hepatic sinusoidal insulin concentrations in the control group tended to decline (from 96 ± 30 pmol/L [baseline] to 68 ± 7 pmol/L [clamp period]; $P = 0.14$), while renal vein plasma insulin concentrations increased in parallel with the rise in arterial insulin. The mean arterial LY concentrations during the final hour (near–steady-state) were 11,651 ± 946, 7,589 ± 843, and 3,906 ± 858 pmol/L in $LY_{0.5}$, $LY_{0.375}$, and $LY_{0.25}$, respectively (Fig. 1B).

Fractional hepatic, intestinal, and renal extractions of regular insulin during the clamp were 50 ± 3%, 29 ± 2%, and 61 ± 3%, respectively. The extraction of LY by the liver could not be precisely quantified because of the high analog concentrations and relatively low tissue clearance. The extraction by the intestinal tract averaged 5 ± 3% at the highest dose, but at the lower LY infusion rates, the analog concentrations were too similar in the artery and portal vein to detect intestinal extraction. In $LY_{0.5}$, renal extraction of the LY was 13 ± 4% (not evaluated in $LY_{0.375}$ and $LY_{0.25}$).
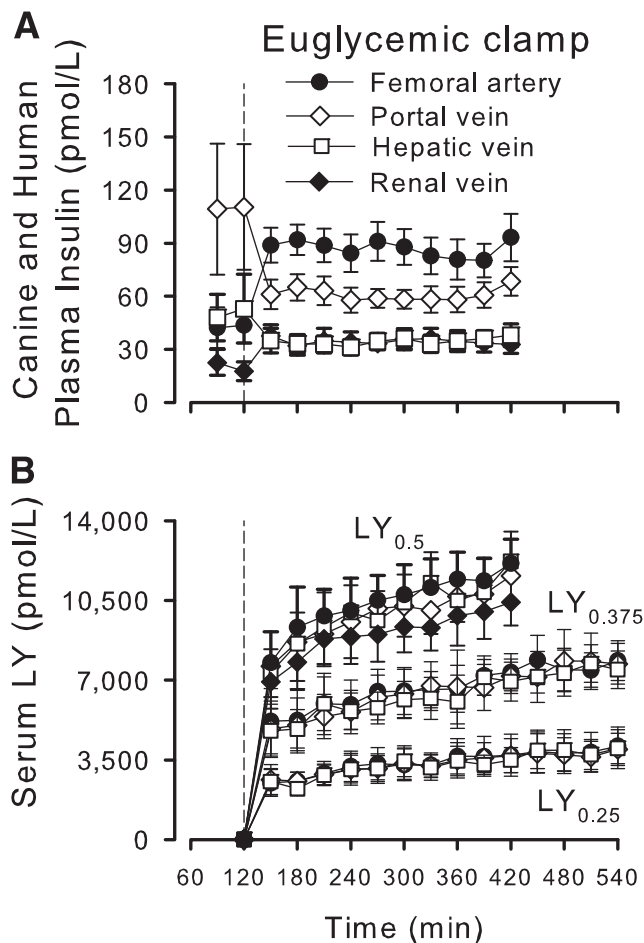
Figure 1—Plasma concentrations of endogenous insulin (basal period) and regular human insulin (clamp period) in control dogs (A) and serum concentrations of LY (B). Arterial, portal vein, hepatic vein, and renal vein (available for control and $LY_{0.5}$ groups only) concentrations of insulin or analog. Data are mean ± SEM; $n = 6$, 6, 5, and 4 for control, $LY_{0.5}$, $LY_{0.375}$, and $LY_{0.25}$, respectively.

Glucose Metabolism

Arterial plasma glucose during the clamp period remained at basal concentrations [~6.1 mmol/L in all groups (Fig. 2A)]. The GIRs were not significantly different between control and $LY_{0.5}$ or $LY_{0.375}$ (Fig. 2B) but were lower ($P < 0.05$) in $LY_{0.25}$ than in control. All groups exhibited net hepatic glucose output (NHGO) (8–11 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) during the baseline period. In the control group, NHGO gradually declined from baseline, reaching approximately zero by 420 min ($\Delta 10.3 \pm 1.4$ $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Fig. 2C). In $LY_{0.5}$, on the other hand, there was a rapid and marked impact at the liver, such that there was a switch from NHGO to net hepatic glucose uptake (NHGU) within 30 min of beginning the $LY_{0.5}$ infusion, and the change from baseline in net hepatic glucose balance reached 16.8 ± 2.2 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ by 420 min ($P < 0.05$ between groups). The change from baseline in $LY_{0.375}$ and $LY_{0.25}$ averaged 18.8 ± 2.6 ($P < 0.05$ vs. control) and 9.5 ± 3.1 (NS vs. control)
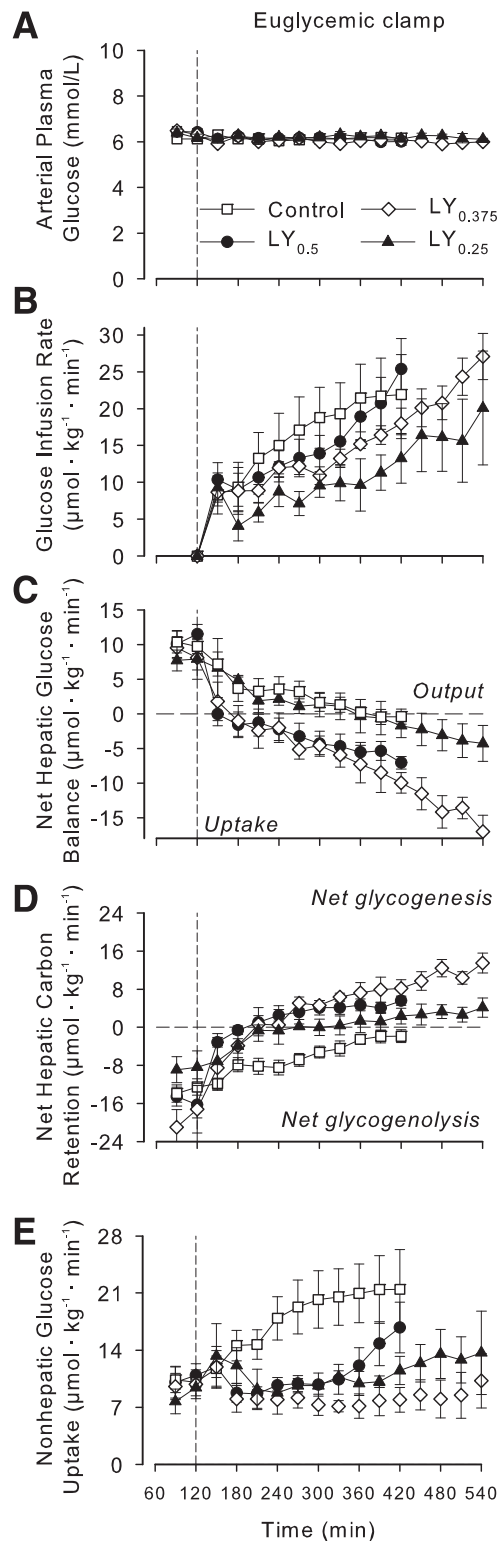
Figure 2—Arterial plasma glucose concentrations (A), GIRs (B), net hepatic glucose balance (NHGB) (C), net hepatic carbon retention (NHCR) (D), and non-HGU (E). Control ($n = 6$), $LY_{0.5}$ ($n = 6$), $LY_{0.375}$ ($n = 5$), and $LY_{0.25}$ ($n = 4$). Significant differences ($P < 0.05$): $LY_{0.5}$ and $LY_{0.375}$ vs. control for NHGB, NHCR, and non-HGU; $LY_{0.25}$ vs. control for GIR, NHCR, and non-HGU; $LY_{0.375}$ vs. $LY_{0.5}$ for non-HGU; $LY_{0.25}$ vs. $LY_{0.5}$ for NHGB, GIR, and NHCR; $LY_{0.25}$ vs. $LY_{0.375}$ for NHGB and NHCR.

$\mu mol \cdot kg^{-1} \cdot min^{-1}$ by 420 min. All LY dosages were associated with significantly greater net hepatic carbon retention than exhibited by the control group (Fig. 2D). The effect of each treatment on endogenous glucose R_a was very similar to its effect on net hepatic glucose balance (Fig. 3A). In the control group, there was a gradual decline in glucose R_a, with the fall from basal totaling $8.9 \pm 1.9 \ \mu mol \cdot kg^{-1} \cdot min^{-1}$ by the final hour. When the change in endogenous glucose R_a between the basal period and 360–420 min (the final hour during part A) was calculated, it was clear that the change was significantly greater in LY$_{0.5}$ and LY$_{0.375}$ than in control (Fig. 3C).

Peripheral insulin infusion in the control group had an immediate impact on nonhepatic glucose uptake (non-HGU), and by the final hour non-HGU had increased $11.1 \pm 4.5 \ \mu mol \cdot kg^{-1} \cdot min^{-1}$ over the basal rate (Fig. 2E). In the LY groups, non-HGU rates initially fell below basal. In the LY$_{0.5}$ group, it began to increase gradually after 330 min, with the increase from basal in the final hour being $4.1 \pm 1.5 \ \mu mol \cdot kg^{-1} \cdot min^{-1}$ ($P = 0.14$ versus control). The LY$_{0.25}$ group also exhibited a small increase in non-HGU during the late part of the clamp, while the rate did not rise above basal at any time in the LY$_{0.375}$ group. In an effort to compare the control and the different LY groups under equivalent conditions, we calculated the ratio of the change from basal in non-HGU to the change in NHGU at a time when GIRs were equivalent in all groups ($\sim20 \ \mu mol \cdot kg^{-1} \cdot min^{-1}$). The lower the ratio in Table 2, the more hepato-preferential the action. The ratio was much higher in control than in LY$_{0.5}$ and LY$_{0.375}$ ($P < 0.01$), and it tended to be higher than in LY$_{0.25}$. No significant differences were seen in either net renal or hindlimb glucose balance between the control and LY$_{0.5}$ groups (data not shown).

LY$_{0.375}$ and LY$_{0.25}$, but not LY$_{0.5}$, stimulated glucose R_d significantly less than regular insulin through 420 min (Fig. 3B). When the ratio of change from baseline in glucose R_d to change in glucose R_a was calculated at the time when GIR was $\sim20 \ \mu mol \cdot kg^{-1} \cdot min^{-1}$ in each group, the control group exhibited a ratio >twofold that in the LY groups (Table 2).

Lactate Metabolism

All groups exhibited net hepatic lactate output (NHLO) during the baseline period, with the rates in LY$_{0.375}$ and LY$_{0.25}$ being significantly higher and lower, respectively, than in the control group (Table 3). During the clamp period, the control group exhibited a slight increase in NHLO followed by a return to the baseline rate. In all of the LY groups, NHLO during the clamp period declined to a nadir no different from $0 \ \mu mol \cdot kg^{-1} \cdot min^{-1}$ in
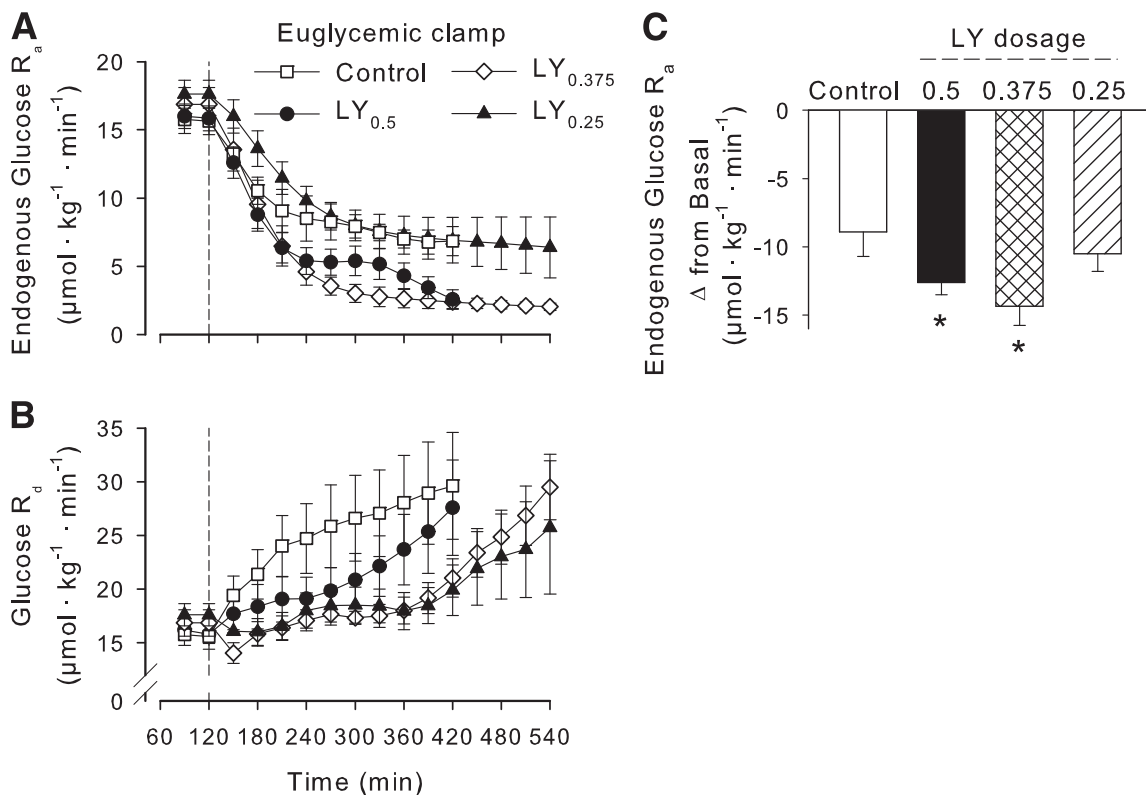
Figure 3—Tracer-determined rate of endogenous glucose R_a (A) and R_d (B). C: Magnitude of the decline in endogenous glucose R_a between the baseline period and 360–420 min (the last hour of study for the control and LY$_{0.5}$ groups). Control ($n = 6$), LY$_{0.5}$ ($n = 6$), LY$_{0.375}$ ($n = 5$), and LY$_{0.25}$ ($n = 4$). $P < 0.05$ for LY$_{0.375}$ vs. control for both R_a and R_d, $P < 0.05$ for LY$_{0.25}$ vs. control for R_d. *$P < 0.05$ vs. control.

TABLE 2—Ratios of change from baseline in non-HGU to change in NHGU, as well as change from basal in tracer-determined glucose R_d to the change in endogenous glucose R_a

Group	Δnon-HGU:ΔNHGU	ΔR_d:ΔR_a
Control	1.17 ± 0.38	1.37 ± 0.32
$LY_{0.5}$	0.09 ± 0.07*	0.60 ± 0.24‡
$LY_{0.375}$	−0.01 ± 0.13*	0.51 ± 0.22‡
$LY_{0.25}$	0.39 ± 0.33†	0.61 ± 0.43‡

Data are mean ± SEM. n = 6/group for control and $LY_{0.5}$; n = 5 for $LY_{0.375}$, and n = 4 for $LY_{0.25}$. Ratios were calculated at the point at which GIR in each group was ~20 μmol · kg^{-1} · min^{-1} (330, 360, 450, and 540 min for control, $LY_{0.5}$, $LY_{0.375}$, and $LY_{0.25}$, respectively). There were no significant differences among the LY groups. *$P < 0.01$ vs. control. †$P = 0.07$ vs. control. ‡$P < 0.05$ vs. control.

$LY_{0.5}$. The $LY_{0.375}$ and $LY_{0.25}$ groups actually switched to net hepatic lactate uptake. NHLO resumed during the latter part of the clamp but did not reach baseline rates by the end of the experiment in any of the LY groups. As a result of these changes, blood lactate concentrations tended to fall initially and then return toward baseline levels in the LY groups.

The control group exhibited net hindlimb lactate uptake throughout the clamp, with uptake increasing significantly as NHLO peaked and then declining to near-baseline rates. Conversely, net hindlimb lactate uptake decreased in $LY_{0.5}$ as NHLO declined, with a subsequent small increase in net limb lactate uptake corresponding to the increase in NHLO by the end of the clamp. Net renal lactate uptake did not change significantly from

baseline in either control or $LY_{0.5}$, and the two groups did not differ at any time point (data not shown).

Glycerol, Fatty Acid, and Triglyceride Metabolism

Arterial glycerol and NEFA concentrations in the control group declined promptly under clamp conditions in response to the peripheral hyperinsulinemia. In contrast, the glycerol and NEFA concentrations initially increased in the LY groups and then gradually declined (Fig. 4A and C). Net hepatic and renal (renal not shown) uptakes of glycerol and NEFA decreased during the clamp in the control group and initially increased in the LY groups, corresponding to the changes in the substrate concentrations (Fig. 4B and D). Net hindlimb glycerol output did not differ between the control and $LY_{0.5}$ groups at any time (data not shown). During the clamp, arterial plasma triglyceride concentrations declined significantly in all groups (Fig. 4E). By 420 min, triglyceride concentrations had fallen ~40% below baseline in the control and $LY_{0.5}$ and $LY_{0.375}$ groups and 15% in $LY_{0.25}$. The concentrations were not significantly different among groups at 420 min.

Tissue Analyses

There was no significant difference between regular insulin and $LY_{0.5}$ in phosphorylation of Akt in the hypothalamus or STAT3 protein in the liver (Fig. 5A and B; Supplementary Fig. 1). On the other hand, phosphorylation of Akt and GSK3β in the liver was significantly enhanced with $LY_{0.5}$ versus regular insulin (Fig. 5C and D).

### DISCUSSION

Insulin secretion into the portal vein results in ~three-fold greater exposure of the liver, versus other tissues, to insulin. The increased exposure of the liver to the

TABLE 3—Lactate data

	Baseline period	Clamp period time (min)						
		180	240	300	360	420	480	540
Arterial lactate (μmol/L)								
Control	701 ± 69	624 ± 72	623 ± 105	623 ± 81	648 ± 73	650 ± 106	—	—
$LY_{0.5}$	771 ± 122	580 ± 83	483 ± 63	503 ± 77	548 ± 93	548 ± 117	—	—
$LY_{0.375}$	952 ± 150*	802 ± 176	552 ± 107	471 ± 41	534 ± 71	602 ± 62	743 ± 68	761 ± 71
$LY_{0.25}$	684 ± 42	551 ± 96	539 ± 73	482 ± 113	498 ± 125	542 ± 84	628 ± 125	887 ± 344
Net hepatic lactate balance (μmol · kg^{-1} · min^{-1})								
Control	6.4 ± 1.9	8.4 ± 0.9	9.8 ± 0.9	7.2 ± 1.0	4.7 ± 1.2	4.6 ± 1.4	—	—
$LY_{0.5}$	9.7 ± 2.9	5.6 ± 1.9	0.6 ± 2.1*	1.4 ± 1.4*	3.0 ± 1.2*	3.5 ± 0.5	—	—
$LY_{0.375}$	20.6 ± 6.7*	9.9 ± 4.1	3.0 ± 4.2	−0.1 ± 1.7*	0.0 ± 1.5*	3.6 ± 1.7	3.4 ± 1.7	7.0 ± 0.9
$LY_{0.25}$	1.7 ± 2.4*	−1.8 ± 2.2*	−2.9 ± 2.2*	−2.6 ± 1.9*	−2.0 ± 1.7*	−1.3 ± 0.7*	−0.4 ± 2.0	0.2 ± 1.3

Data are mean ± SEM. n = 6, 6, 5, and 4 for control, $LY_{0.5}$, $LY_{0.375}$, and $LY_{0.25}$, respectively. Basal period values are the mean of two sampling times; the clamp period took place between 120 and 420 min (for control and $LY_{0.5}$) and between 120 and 540 min (for $LY_{0.375}$ and $LY_{0.25}$). Positive values for balance indicate net hepatic output. There were no significant differences among the LY groups; $P = 0.1$ for basal period net balance in $LY_{0.375}$ compared with the rates in $LY_{0.5}$ and $LY_{0.25}$. *$P < 0.05$ vs. control.

A

Euglycemic clamp


B

C

D

E

Time (min)
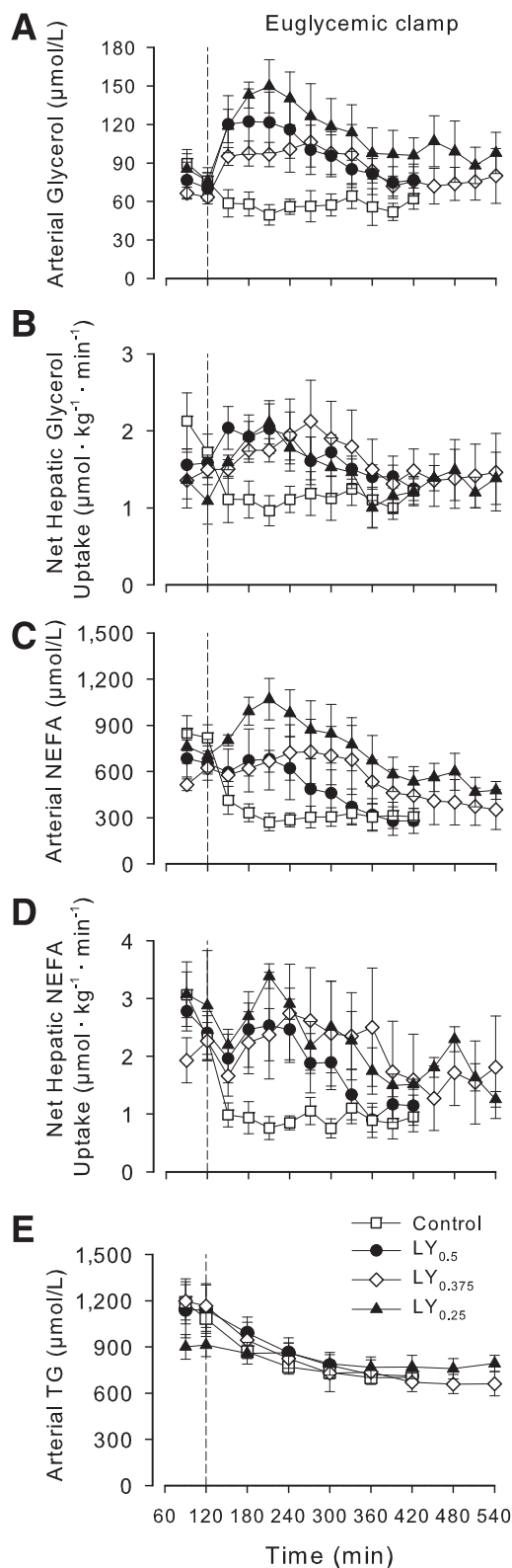
Figure 4—Arterial blood glycerol (A), plasma NEFA (C), and plasma triglyceride (TG) (E) concentrations and net hepatic uptakes of glycerol (B) and NEFA (D). Control (n = 6), LY$_{0.5}$ (n = 6), LY$_{0.375}$ (n = 5), and LY$_{0.25}$ (n = 4). $P < 0.05$ for all LY groups vs. control for arterial glycerol and NEFA and for net hepatic uptake of glycerol and NEFA. There were no significant differences among groups in TG concentrations. There were no significant differences among LY groups for any parameter.

hormone results from its secretion into a limited vascular pool and the fact that the liver extracts 50–60% of the insulin to which it is exposed (24). In contrast, when insulin is administered subcutaneously, the normal portal-peripheral insulin gradient is lost so that the peripheral tissues are comparatively hyperinsulinemic while the liver is comparatively hypoinsulinemic (25,26). The portal vein, hepatic vein, and hepatic sinusoidal insulin concentrations in the control group during the clamp period were ~30–40% below basal, while the arterial concentrations were approximately two times basal. Although the arterial concentrations did not increase as much as would be anticipated after a mixed meal (six-fold basal or more), they were inappropriately high in relation to the other blood vessels sampled, which also exhibit sixfold or more increases in insulin after meal ingestion (27). Partly as a result of the relative over-insulinization of peripheral tissues, even individuals with well-controlled insulin-treated diabetes are apt to experience glycemic fluctuations, hypoglycemia, and dyslipidemia, which have been suggested to contribute to microvascular and macrovascular complications (28). Theoretically, insulin analogs with hepato-preferential actions could reduce the risk of these complications (29,30).

While it may seem counterintuitive that a hepato-preferential insulin could reduce hypoglycemia, since the liver's response is key to restoring euglycemia, relative overinsulization of peripheral tissues contributes to hypoglycemia and impaired counterregulation. Lipolysis is an important contributor to gluconeogenesis under hypoglycemic conditions, and the relative overinsulinization of the peripheral lipid depots resulting from current peripherally administered insulin therapies would be expected to impede gluconeogenesis (31–33). Moreover, even though skeletal muscle glucose uptake decreases during hypoglycemia associated with conventional insulin therapy, it does not cease. Due to the relatively large mass of skeletal muscle, this results in significant ongoing glucose disposal, a process that would be curtailed by a hepato-preferential insulin. More importantly, a hepato-preferential insulin puts the primary burden of hypoglycemia on the organ best suited to cope because hepatic insulin signaling is markedly reduced in the presence of hypoglycemia; i.e., the insulin receptor is essentially disengaged (34). Even when overinsulinized and deprived of increases in counterregulatory hormones and central neural stimuli, the liver is capable of responding directly to hypoglycemia by increasing its glucose output (35–37).

At the doses examined, LY exhibited a clear hepato-preferential effect compared with human insulin when both products were delivered via peripheral vein. This was demonstrated by two independent measures of glucose metabolism: organ balance and tracer-determined turnover. It should be noted that a single control group was used as a comparator for all three LY dosages. In
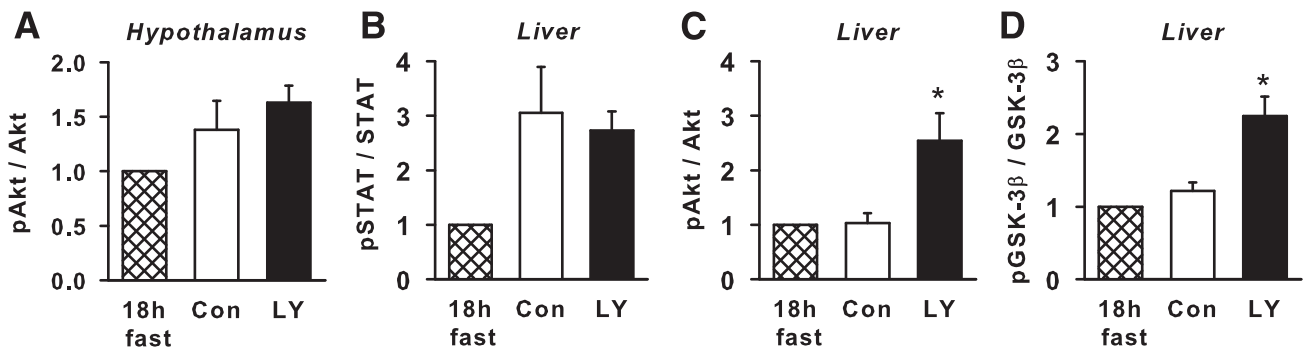
Figure 5—Molecular markers of insulin signaling in the control and $LY_{0.5}$ groups. Hypothalamic phosphorylated Akt (pAkt), expressed relative to total Akt (A), as well as hepatic pSTAT3/STAT3 (B), pAkt/Akt (C), and pGSK3β/GSK3β (D), in control (con) (white bars) and $LY_{0.5}$ (black bars) (n = 6/group). The hatched bars are mean data from 2 additional dogs, not included in the experiments, that were fasted for 18 h and then killed to provide reference (non–insulin stimulated) tissue. See Supplementary Fig. 1 for representative blots. *P < 0.05 vs. control.

order for groups to be compared, in terms of glucose disposition, they must be matched for at least one parameter of interest. In that regard, the $LY_{0.5}$ and $LY_{0.375}$ groups were not significantly different in GIR from the control group (P = 0.72 and 0.35, respectively). Both $LY_{0.5}$ and $LY_{0.375}$ exhibited a faster and greater effect on net hepatic glucose balance than regular insulin, quickly shifting the liver from NHGO to NHGU. Conversely, their effect on non-HGU was delayed and minimal compared with regular insulin. At the lowest LY dose, the analog's effects on net hepatic glucose balance were comparable to those in the control group throughout 420 min (P = 0.974), making possible a comparison of nonhepatic effects in the presence of similar rates of NHGO. The impact of $LY_{0.25}$ on non-HGU was markedly lower than that of regular insulin. Of the three LY dosages examined, $LY_{0.375}$ appeared to have the greatest effect at the liver concomitant with the least effect on nonhepatic tissues.

Tracer methodology yielded results consistent with hepatic glucose balance data. The two higher doses of LY caused a significantly greater fall in endogenous glucose R_a than regular insulin (even though significant differences from control in the rates were not evident as early as they were with organ balance data), while the effect of $LY_{0.25}$ was very similar to that of regular insulin (P = 0.34). On the other hand, both $LY_{0.375}$ and $LY_{0.25}$ stimulated glucose R_d significantly less than regular insulin. Moreover, all doses of LY, compared with regular insulin, were associated with a significantly smaller change in glucose R_d relative to the impact on endogenous R_a.

In contrast to its rapid effects on the liver, as noted above, LY's effect on nonhepatic tissues was more gradual than that of regular insulin. In particular, LY's effect on fat metabolism appeared to be slow. Lipolysis is extremely sensitive to an elevation of regular insulin, and thus it was quickly suppressed in the control group, with glycerol concentrations reaching a stable nadir within 30 min of the start of the clamp. In none of the LY groups did the glycerol concentrations fall below the

baseline values at any time, suggesting a lack of lipolytic inhibition by the analog at these doses. NEFA levels, on the other hand, fell below baseline levels during the clamp in all LY groups, consistent with an increase in reesterification. Nevertheless, regular insulin lowered NEFA much more promptly than LY. The nadir of NEFA concentrations (271 ± 57 μmol/L) occurred within 90 min after the start of the clamp period in the control group, while the nadir in $LY_{0.5}$ (278 ± 92 μmol/L) was not reached until 270 min after the start of the clamp, and $LY_{0.375}$ and $LY_{0.25}$ did not reach their lowest concentrations (353 ± 130 and 467 ± 96 μmol/L, respectively) until the last hour. An insulin-induced fall in circulating NEFA provides an indirect means for reducing hepatic glucose production (38,39), and thus the sluggish response of glycerol and NEFA concentrations to LY administration suggests that it was the direct effect of the analog that inhibited hepatic glucose production.

A decrease in NEFA levels and hepatic NEFA uptake is associated with an increase in hepatic glycolysis (39). The exact mechanism for this is unclear, but it may well result from a fall in intrahepatic citrate, one of the major inhibitors of phosphofructokinase, the first rate-determining enzyme in the glycolytic pathway. Alternatively, the fall in NEFA could alter the intrahepatic redox state, increasing the NADH-to-NAD ratio and leading to an activation of lactate dehydrogenase. (For further discussion, see Sindelar et al. [39]) Thus, when net hepatic NEFA uptake decreased in the control group, there was an increase in the release of glycolytic carbon (lactate) from the liver. Conversely, there appeared to be a prompt suppression of glycolysis (hepatic lactate release) in response to LY, likely related to the increase in lipolysis early in the experimental period. The two lower rates of LY infusion not only reduced NHLO but also actually prompted the liver to shift to net uptake of lactate, consistent with increased storage of carbon as glycogen in the liver.

The liver is a key organ in the extraction of regular insulin, but in contrast, hepatic extraction of LY

appeared to be very low. As with other slowly cleared insulin analogs, such as detemir (40), the plasma LY concentrations were high relative to those of regular insulin. However, it is evident that we achieved three distinct LY levels and near–steady-state concentrations, particularly with the longer studies in Part B. With such high circulating concentrations and low clearance, it is difficult to quantify tissue LY extraction precisely. Even though the kidney was an important site of extraction for LY, renal LY extraction was proportionally much less than with human insulin, as designed (9). When LY was tested in subjects with renal impairment (ranging from mild to end-stage renal disease), the apparent clearance and half-life of the drug did not differ from that in subjects with normal renal function (41). Thus, the pharmacokinetic properties of LY do not appear to be affected by impairment in renal function.

Because of the large hydrodynamic size of LY, it might not be expected to cross the blood-brain barrier (42). Nevertheless, hypothalamic Akt phosphorylation at the end of the experiment was not different in the control and LY-treated animals. The affinity of LY for the insulin receptor, although low (<6% of that of insulin lispro [43]), might permit some passage into the central nervous system (CNS) via saturable mechanisms. Additionally, there is a potential for LY to reach the CNS via extracellular pathways such as are used by some very large molecules, e.g., albumin and certain antibodies (44–46). The current findings are not unique or completely unexpected, since the insulin analog detemir (which is tightly bound to serum albumin [47]) has been reported not to cross the mouse blood-brain barrier (48), and yet it has a more rapid and pronounced effect on hypothalamic insulin receptor phosphorylation than human insulin when both are injected intravenously in mice (49). The similarity of phosphorylated STAT3/STAT3 in the livers of the control and $LY_{0.5}$ groups is consistent with an effect of LY in the CNS, since hepatic STAT3 phosphorylation has been suggested to be a marker of brain insulin signaling (50,51) that can be blunted with infusion of an ATP-sensitive K^+-channel inhibitor or a phosphatidylinositol-3 kinase inhibitor into the 3rd cerebroventricle of the dog (51). Nevertheless, hepatic STAT3 phosphorylation in response to brain insulin signaling occurs slowly also (50–52), affecting gene transcription slowly, and thus could not have been involved in bringing about the rapid response effect on liver glucose metabolism observed with LY infusion.

The significant stimulation of hepatic Akt and GSK3β phosphorylation in the LY-treated versus control animals is in keeping with the hepato-preferential metabolic actions of the analog. Conversely, the lack of apparent stimulation of phosphorylation of these proteins by regular insulin (in view of the data from the 18-h-fasted, non–insulin-treated animals) is in keeping with the lack of increase in hepatic insulin concentrations during the clamp; indeed, the hepatic sinusoidal insulin

concentrations were below basal in the control group. GSK3β is inactivated by phosphorylation, thus allowing glycogen synthesis to proceed. Consistent with this, net hepatic carbon retention, a good surrogate measure for hepatic glycogen synthesis (53), was enhanced in the LY-treated groups.

The physiologic basis of the hepato-preferential effect of LY is not fully understood but is likely related to its large hydrodynamic size. While this may impede passage from the capillaries into the interstitium of peripheral target tissues, i.e., muscle and adipose, the hepatic sinusoidal capillaries are fenestrated, with openings of 100–200Å in diameter, allowing entry of larger molecules into the organ (e.g., lipoproteins), with many factors affecting hepatic clearance (54,55).

In conclusion, LY at all dosages examined exhibited a more hepato-preferential effect than regular human insulin when both products were delivered by peripheral vein. The effect on the liver was evident throughout the duration of the clamp period, with the use of both tracer and organ balance techniques. If these effects can be sustained during long-term dosing, peripherally administered LY appears to have the potential to reproduce the hepato-preferential effects of endogenously secreted insulin—an effect that might reduce complications associated with current insulin therapy.

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