Simulated first-phase insulin release using Humulin or insulin analog HIM2 is associated with prolonged improvement in postprandial glycemia

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insulin secretion in countering the action of glucagon on glucose turnover in the presence of fourfold-elevated glucagon. Theses studies revealed that simulated first-phase insulin was more effective than second-phase in countering the glucagon effect.

The present study had two aims. The first was to examine the ability of first-phase insulin release to modify net hepatic glucose uptake under conditions mimicking oral glucose loading in an insulinoopenic state and to assess its potential prolonged effect on glucose uptake by nonhepatic tissues. The second aim was to determine whether a pulse of an insulin analog (hexyl-insulin monoconjugate 2, HIM2, Nobex), created to facilitate the absorption of orally delivered insulin, would have the same ability to modify postprandial hepatic glucose metabolism as Humulin given at the same rate. In this way, the pharmacokinetics and pharmacodynamics of the molecules could be compared in the absence of the variability caused by gut absorption of HIM2.

RESEARCH DESIGN AND METHODS

Animals and surgical procedures. Studies were carried out on conscious 42-h-fasted mongrel dogs with a mean weight of 23.1 ± 0.4 kg. A fast of this duration was chosen because it results in liver glycogen levels that are at a stable minimum (~20 mg/g liver) (12).

Approximately 16 days before study, each dog underwent a laparotomy under general anesthesia (15 mg/kg pentothal sodium presurgery and ~1% isoflurane inhalation anesthetic during surgery), and silicone rubber catheters were inserted for sampling in the hepatic vein, the portal vein, and a femoral artery, as described in detail elsewhere (22). Catheters for hormone infusion were also placed in a splenic and a jejunal vein. A duodenal catheter was inserted for glucose infusion (19). Transonic flow probes (Transonic Systems, Ithaca, NY) were placed around the portal vein and the hepatic artery. The catheters were filled with saline containing heparin (1,000 U/ml; Abbott Laboratories, North Chicago, IL), their free ends were knotted, and they, along with the free ends of the transonic leads, were placed in two separate subcutaneous pockets. Diet, housing, protocol approval, criteria for study, and preparation for study were as previously described (19). This protocol was approved by Institutional Animal Care and Use Committee at Vanderbilt University, Nashville, TN.

Experimental design. Each experiment consisted of a 100-min equilibration period (~140 to ~40 min), a 40-min basal period (~40 to 0 min), and a 270-min experimental period (~270 min). In all experiments, a constant infusion of indocyanine green (ICG) dye (0.076 mg/min) was initiated at ~140 min. At 0 min, a constant infusion of somatostatin (SRIF; 0.5 μg·kg⁻¹·min⁻¹) was begun to suppress endogenous insulin and glucagon secretion, and glucagon (0.55 ng·kg⁻¹·min⁻¹) and human insulin (0.25 μU·kg⁻¹·min⁻¹) were replaced intraportally to maintain basal levels in each protocol. The rate of SRIF infusion was lower than that used in past studies so that the inhibitory effect of the peptide on gastric emptying seen with higher SRIF doses could be eliminated. This rate of SRIF infusion did not affect gastric motility, as indicated by the fact that ~98% of the intraduodenal glucose that was infused was absorbed, and yet both the insulin and glucagon levels were successfully clamped. Thus a somatostatin rate of 0.5 μg·kg⁻¹·min⁻¹ was adequate to clamp the pancreatic hormones while not impeding glucose absorption.

In the first group, insulin was infused into the portal vein at a basal rate of 0.25 mU·kg⁻¹·min⁻¹ throughout the study (BI, n = 6). In the second group, a bolus of human insulin was given into the portal vein from 0 to 5 min at an infusion rate of 10 mU·kg⁻¹·min⁻¹ to mimic first-phase insulin release postprandially (HI, n = 6) followed by basal insulin infusion as above. In the third group, HIM2 (an orally active, modified insulin created by Nobex, Research Triangle Park, NC) was infused into the portal vein at 10 mU·kg⁻¹·min⁻¹ (HIM2, n = 6) over 5 min as a bolus followed by a basal infusion of human insulin as above. Glucose (50% dextrose) was infused peripherally at 5 mg·kg⁻¹·min⁻¹ from 5 to 10 min and then at 10 mg·kg⁻¹·min⁻¹ from 10 to 15 min to prevent hypoglycemia from occurring. At 15 min, the duodenal glucose (20% dextrose) infusion was started and continued at 5 mg·kg⁻¹·min⁻¹ for the duration of the experiment. The purpose of the 15-min delay in starting the duodenal glucose infusion was to simulate insulin dosing before a meal.

Femoral artery, portal vein, and hepatic vein blood samples were taken every 20 min during the basal period (~40 to 0 min) and every 30 min after ~t = 30 min. The arterial and portal vein samples were taken simultaneously, and hepatic vein samples were collected ~30 s later to compensate for transit time of glucose through the liver. The total volume of blood withdrawn did not exceed 20% of the animal’s blood volume, and 2 volumes of normal saline were infused for each volume of blood withdrawn.

After completion of each experiment, the animal was killed with an overdose of pentobarbital, and a tissue sample from three of the seven liver lobes and from muscle was rapidly frozen with clamps kept in liquid nitrogen. The tissue was stored at ~70°C for later analysis of glycogen.

Processing and analysis of samples. The collection and immediate processing of blood samples have been described previously (9). Four to eight 10-μl aliquots of plasma from each sample were immediately analyzed for glucose via the glucose oxidase technique using a glucose analyzer (Beckman Instruments, Fullerton, CA). Plasma insulin and glucagon concentrations were determined by RIA, as previously described (13). A comparison of standard curves was performed using Humulin and HIM2. The two curves were similar but not identical, having slightly different ED50 values. When human insulin standards were used, the assay slightly underestimated (5%) HIM2 values at the high end of the standard curve (~150 μU/ml) and slightly overestimated (20%) them at the lower end of the curve (~10 μU/ml). At the mid points of the curves, the values were similar. Samples were diluted when necessary to achieve a value near the mid point of the curve.

Free fatty acids (FFAs), cortisol, lactate, and glycerol were measured as previously described (4, 17).

Calculations and data analysis. Hepatic blood flow was measured using transonic flow probes (Transonic System, Ithaca, NY) and by the use of ICG according to the method of Leevy et al. (16). The results obtained with the Transonic flow probes and ICG were not significantly different, but the data reported here were calculated using the Transonic-determined flow because this measurement did not require an assumption regarding the distribution of the arterial and portal vein contribution to hepatic blood flow.

The hepatic substrate load, net hepatic balance, net fractional hepatic extraction, and hepatic sinusoidal insulin and glucagon concentrations were calculated as described previously, using direct calculations for glucose balance (24). Nonhepatic glucose uptake was calculated by adding the net hepatic glucose balance to the average net gut balance and subtracting for any change in the glucose mass between the two time points in question. Nonhepatic glucose clearance was calculated by dividing the nonhepatic glucose uptake by the average arterial blood glucose concentration between the two time points in question.

Glucose absorption was calculated by taking the difference between net gut glucose output during the experimental period and the average basal net gut glucose uptake adjusted for the increase in the arterial plasma glucose level. The latter was calculated as the arterial plasma glucose concentration during the last hour of the study divided by the average basal arterial plasma glucose concentration multiplied by the basal gut glucose uptake rate multiplied by a correction factor of 0.8 as previously determined (21).

For all glucose balance calculations, glucose concentrations were converted from plasma to blood values by using previously deter-
Glucose output averaged 3.7 µg/kg/min in the group given the pulse of the insulin analog (HIM2) spikes (HIM2) were created on the background of basal insulin (BI), but the glycemic profile was markedly different. The rise in arterial plasma glucose occurred more slowly, eventually reaching a plateau of only 193 ± 14 mg/dl during the last hour of the study. Despite very similar glucose absorption rates, the glycemic profiles in the three groups were different. The rise in arterial plasma glucose occurred more slowly, eventually reaching a plateau of only 193 ± 14 mg/dl during the last hour of the study. Despite very similar glucose absorption rates, the glycemic profiles in the three groups were different.

Statistical analysis. All data are presented as means ± SE. Time course data were analyzed with repeated-measures analysis of variance. Independent t-tests were used for any comparisons of mean data. Statistical significance was accepted at *P < 0.05.

RESULTS

Plasma glucagon and insulin concentrations. Arterial and liver sinusoidal plasma glucagon levels were basal throughout the study in all three groups (Table 1). The arterial and liver sinusoidal plasma insulin levels in the BI group remained basal (6 ± 1 and 18 ± 2 µU/ml, respectively) throughout the experiment (Fig. 1). The arterial plasma insulin level in the HI group rose rapidly and peaked at 52 ± 15 µU/ml, whereas the liver sinusoidal insulin level peaked at 171 ± 66 µU/ml; these levels returned to baseline by 30 min. The arterial and liver sinusoidal plasma insulin levels in the HIM2 group peaked at 164 ± 44 and 427 ± 185 µU/ml, respectively. These values did not return to baseline until ~1 h.

Net gut balance, glucose absorption, and arterial glucose level. Net gut glucose uptake was similar in all three groups during the control period and averaged 0.50 ± 0.05 mg·kg⁻¹·min⁻¹. The switch from net gut glucose uptake to net glucose output occurred ~10 min after the initiation of the intraduodenal glucose infusion in all three groups. Net gut glucose output averaged 3.7 ± 0.4 mg·kg⁻¹·min⁻¹ over the study period (Fig. 2).

Glucose absorption occurred at a steady state (4.9 ± 0.8 mg·kg⁻¹·min⁻¹) in the BI group and caused the plasma glucose level to rise to a plateau of 265 ± 20 mg/dl (Fig. 2) during the last hour of the study. At steady state, glucose absorption accounted for 98% of the infused glucose; the remaining 2% was presumed to be metabolized by the gut.

In the HI group, glucose absorption was similar (4.8 ± 0.5 mg·kg⁻¹·min⁻¹) to that seen in the BI group. In this case, however, the arterial plasma glucose rose less, reaching only 214 ± 15 mg/dl by the last hour of the study. Glycose absorption in the group given the pulse of the insulin analog (HIM2) was identical to that in the other two groups (4.9 ± 0.5 mg·kg⁻¹·min⁻¹), but the glycemic profile was markedly different. The rise in arterial plasma glucose occurred more slowly, eventually reaching a plateau of only 193 ± 14 mg/dl during the last hour of the study. Despite very similar glucose absorption rates, the glycemic profiles in the three groups were different.

Table 1. Arterial and liver sinusoidal glucagon levels and percentages of glucose load extracted by the liver during basal and experimental periods in BI, HI, and HIM2 groups in conscious 42-h fasted dogs

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Basal</th>
<th></th>
<th></th>
<th>Experimental Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial plasma glucagon, pg/ml</td>
<td>BI</td>
<td>37±4</td>
<td>37±5</td>
<td>34±7</td>
</tr>
<tr>
<td>Liver sinusoidal plasma glucagon, pg/ml</td>
<td>BI</td>
<td>42±7</td>
<td>42±10</td>
<td>42±8</td>
</tr>
<tr>
<td>Liver sinusoidal plasma glucagon, %</td>
<td>BI</td>
<td>15±0.3</td>
<td>2.7±1.2</td>
<td>2.4±1.1</td>
</tr>
<tr>
<td></td>
<td>HI</td>
<td>3.0±1.3</td>
<td>1.5±1.0</td>
<td>2.8±0.6</td>
</tr>
<tr>
<td></td>
<td>HIM2</td>
<td>2.7±3.9</td>
<td>3.1±2.6</td>
<td>2.1±1.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. BI, basal insulin; HI, human insulin (Humulin); HIM2, insulin analog hexyl-insulin monoconjugate 2. Blanks in net hepatic fractional extraction denote no net glucose extraction.
markedly different, indicating that the insulin spike augmented disposition of the absorbed glucose and resulted in glycemic improvement even 4.5 h later.

Hepatic glucose load, net hepatic glucose balance, and hepatic fractional glucose extraction. Average hepatic blood flows were similar among the three groups. In the BI group, the total blood flow was 33 ± 3 ml·kg⁻¹·min⁻¹, whereas in the HI and HIM2 groups it was 36 ± 3 and 31 ± 4 ml·kg⁻¹·min⁻¹, respectively. At the end of the experiment, the hepatic glucose loads were 70 ± 5, 60 ± 1, and 50 ± 7 mg·kg⁻¹·min⁻¹ in the BI, HI, and HIM2 groups, respectively (P < 0.05 in BI vs. HIM2; Fig. 3).

During the basal period net hepatic glucose output was similar in all three groups (~1.7 ± 0.1 mg·kg⁻¹·min⁻¹). In the BI group, it did not decrease significantly until 90 min after the initiation of glucose absorption. By the last hour of the study, however, net hepatic glucose uptake occurred at the rate of 2.7 ± 0.7 mg·kg⁻¹·min⁻¹. Net hepatic glucose output declined more rapidly in the HI group, ceasing by 60 min. It declined most rapidly in the HIM2 group, in which it ceased at 30 min. By the last hour of the study, net hepatic glucose uptake was 2.7 ± 0.6 and 2.0 ± 0.3 mg·kg⁻¹·min⁻¹ in the HI and HIM2 groups, respectively (Fig. 3). At that time, the fractional extraction of glucose by the liver was ~3–5% in all three groups (Table 1).

Nonesterified fatty acids and glycerol. In the BI group, glycerol levels and net hepatic glycerol uptake fell slightly and eventually plateaued (Fig. 4). In the HI group, the glycerol levels and net hepatic glycerol uptake fell rapidly in the first 15 min, then rose slightly, and then drifted down. In the HIM2...
group, the glycerol levels and the net hepatic glycerol uptake fell rapidly over the first 30 min, reached a minimum at 60 min, and then drifted up slightly. By the end of the experiment, there were no significant differences in glycerol levels or net hepatic glycerol uptake among the three groups. In general, the plasma nonesterified fatty acid (NEFA) levels (Fig. 5) and net hepatic NEFA uptakes paralleled the changes in glycerol.

Lactate levels and hepatic balance. The liver exhibited net lactate uptake in the basal periods in all three groups, and hyperglycemia resulted in a switch to net hepatic lactate output (Fig. 6). At the end of the experiment, net hepatic lactate output was $11.2 \pm 4.4$, $8.8 \pm 1.4$, and $5.4 \pm 3.2 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in the BI, HI, and HIM2 groups, respectively. The arterial blood lactate levels steadily rose in all three groups due to the switch in net hepatic lactate balance and reached an average value of 1,500 $\mu$mol/l by the end of the experiment.

Nonhepatic glucose uptake and nonhepatic glucose clearance. By the end of the experiment, the HIM2 pulse was associated with a significantly greater nonhepatic glucose clearance ($1.61 \pm 0.29 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) than basal insulin (BI = $0.62 \pm 0.11 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or the Humulin pulse (HI = $0.76 \pm 0.26 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; Fig. 7). These differences in nonhepatic glucose clearances are also reflected in the nonhepatic glucose uptake values (Table 2).

DISCUSSION

These data indicate that simulation of first-phase insulin release during an intraduodenal glucose infusion, given on the background of a pancreatic clamp, resulted in a significant improvement of the glycemic profile for as long as 4 h. We also showed that a modified insulin (HIM2) molecule improved glycemia even more than Humulin, probably as a result of its modified pharmokinetics. It would appear that a pulse of insulin improves postprandial glycemia by two different mechanisms. The early blunting of the glycemic rise is mediated by a quick increase in net hepatic glucose uptake, whereas the later improvement is associated with an increase in nonhepatic glucose clearance.

After the 5-min pulse of human insulin, arterial plasma insulin levels rose rapidly and remained elevated for 30 min. Administration of HIM2 caused an elevation in plasma insulin levels that lasted almost 60 min. Despite infusion of the same amount of insulin in both groups, the HIM2 spike created a significantly higher arterial plasma insulin level than did the human insulin spike. The area under the curve (AUC) for HIM2 was almost three times greater than that seen in the HI group. These data suggest that clearance of the insulin analog was significantly reduced relative to that of Humulin. HIM2 clearance was undoubtedly altered as a result of the structural modification of the molecule. Its structure is composed of a single amphipathic oligomer of low molecular weight covalently linked to a free amino group on the Lys-B29 residue of human insulin.
recombinant human insulin via an amide bond. It is readily absorbed due to its amphiphilic nature and has enhanced resistance to degradation by enzymes, such as insulin protease, (8), which is unable to reach its sites of action due to steric interference (28). It is clear that the biological activity of HIM2 insulin was reduced relative to Humulin, because the threefold increase in the AUC for plasma insulin improved glucose kinetics only slightly. The fact that HIM2 brought about a slight improvement in glycemia, relative to Humulin, suggests that the decrease in its biological action was not exactly proportional to its altered clearance.

Clearly any improvements in glycemia seen in response to the pulse of insulin cannot be due to the difference in glucose delivery, since all three groups received glucose intraduodenally at the same rate. Furthermore, the calculated glucose absorption rate was identical in all three groups. Therefore, the difference in the arterial plasma glucose levels must be attributed to changes in glucose utilization. In the early part of the response (15–120 min), the liver ceased glucose production and began to take up glucose most rapidly in the HIM2 group. There are three major factors that control net hepatic glucose uptake: the glucose load to the liver, the insulin level, and the portal signal (i.e., generated when the plasma glucose concentration in the portal vein is higher than the arterial plasma glucose concentration). In these experiments, the portal signal was the same in all three groups; thus the differences in net hepatic glucose uptake must be related to the glucose load and/or the insulin concentration (23). The increased hepatic response in the Humulin group must have been attributable to the increase in insulin, because the hepatic glucose loads over the first 2 h in the BI and HI groups were equal. In the HIM2 group, the hepatic glucose load was less than that in the BI group, but the insulin spike was much larger. Clearly, therefore, the latter was responsible for the more rapid increase in net hepatic glucose uptake seen in this group.

At the end of the experiment, there were minimal differences between the three groups in either net hepatic glucose balance or hepatic fractional glucose extraction. The rate of net hepatic glucose uptake was slightly less with HIM2 as a result of the decreased hepatic glucose load. Nevertheless, differences in net hepatic glucose uptake cannot explain the prolonged improvement that the insulin spikes had on the arterial plasma glucose level; instead, it appears to be attributable to the effect of insulin on nonhepatic tissues. This is somewhat surprising, given that the plasma insulin levels returned to baseline no later than 60 min after dosing even in the HIM2 group. One possible explanation for the finding, however, is that the insulin levels in the interstitial fluid may still be elevated at these latter time points, as suggested by Getty et al. (11). Additionally, altered insulin kinetics in the interstitial fluid may have prolonged the effect of HIM2 on nonhepatic tissues even further. In agreement with this, nonhepatic glucose clearance in the HIM2 group was significantly greater than it was in the BI group. Nonhepatic glucose clearance in the HI group, on the other hand, was only slightly greater than that seen in the BI group.

FFAs are known to alter muscle and liver glucose metabolism. There exists an inverse relation between plasma FFA concentrations and insulin sensitivity (25). In normal individuals, an increase in FFAs has been shown to cause hepatic insulin resistance by interfering with the normal suppression of glycogenolysis by insulin (2). Insulin resistance in skeletal muscle can also be attributed to an increase in fatty acids. Type 2 diabetics who are given nicotinic acid to induce a decrease in plasma FFA levels have a decrease in glucose production with no compensatory increase in glycogen synthesis; this leads to an overall decrease in endogenous glucose production (1). It has been shown (26) that hepatic glucose production can be suppressed by an increase in peripheral insulin, partly due to an inhibition of lipolysis. We have shown in the dog that FFA levels control the glycolytic flux in the liver (20). A fall in FFA levels and, as a result, in the net hepatic uptake of FFA directs intrahepatic carbon into glycolysis, eventually giving rise to lactate (26). Given that the FFAs fall most dramatically in the HIM2 group and substantially in the HI group, the early differences in net hepatic glucose output might be attributable, in part, to changes in plasma FFA levels. Wajcberg et al. (29) have suggested that HIM2’s prolonged biological action may be attributed to a persistent suppressive effect on FFA. There may be a prolongation of antilipolysis due to an enhancement of HIM2 binding to the insulin receptors in the adipocytes. Unless these early changes in FFAs had a prolonged effect on glucose uptake by muscle, it seems unlikely that they were affecting the glucose profile toward the end of the experiments, since the plasma FFA levels were similar in all three groups by then.

In summary, a brief 5-min pulse of insulin infused intraportally at a rate of 10 mU·kg⁻¹·min⁻¹ simulated the first-phase insulin release seen postprandially, increased plasma insulin levels for 30 min, and eliminated net hepatic glucose production by 60 min. It still had a significant effect on plasma glucose levels 4.5 h later. HIM2 was cleared less efficiently than Humulin, resulting in higher plasma insulin levels at both the liver and the periphery. This pulse increased plasma insulin levels for almost 60 min, eliminated net hepatic glucose production by 30 min, and continued to have a significant effect on plasma glucose levels even at the end of the experiment (4.5 h). These data demonstrate the ability of a brief burst of insulin secretion to result in prolonged glycemic improvement and

<table>
<thead>
<tr>
<th>Time, min</th>
<th>BI 1.4±0.4</th>
<th>BI 1.2±0.3</th>
<th>HIM2 1.1±0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhepatic glucose uptake, mg·kg⁻¹·min⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0−15</td>
<td>2.3±0.4</td>
<td>2.2±0.4</td>
<td>2.5±0.3*</td>
</tr>
<tr>
<td>15−30</td>
<td>3.3±0.4</td>
<td>2.2±0.5*</td>
<td>2.5±0.3*</td>
</tr>
<tr>
<td>30−60</td>
<td>2.6±0.5</td>
<td>1.6±0.5</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>60−90</td>
<td>3.2±0.7</td>
<td>2.0±0.9</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>90−120</td>
<td>2.6±0.5</td>
<td>1.6±0.8</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>120−150</td>
<td>2.1±0.5</td>
<td>1.7±0.3</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>150−180</td>
<td>1.8±0.5</td>
<td>1.4±0.4</td>
<td>2.3±0.5*</td>
</tr>
<tr>
<td>180−210</td>
<td>1.3±0.3</td>
<td>1.2±0.4</td>
<td></td>
</tr>
<tr>
<td>210−270</td>
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Data are means ± SE. *Significant statistical difference from BI (P < 0.05). ‡Significant statistical difference from BI (P < 0.05).
speak to the importance of first-phase insulin release in post-prandial glucose homeostasis. They also point to the need to further evaluate the pharmacokinetic and pharmacodynamic properties of the HIM2 insulin analog. Subsequent studies are needed to compare the biological activities of HIM2 and Humulin at selective tissues (muscle, fat, liver) within the body. Although several studies (6, 15, 29) have shown that the HIM2 analog is effective in the human following oral delivery, our data suggest that a better understanding of its tissue-specific effects is needed in view of its decreased biological activity and clearance.

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