Hepatic glucose production is more sensitive to insulin-mediated inhibition than hepatic VLDL-triglyceride production

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THE LIVER IS A VERY IMPORTANT REGULATOR in the homeostasis of both glucose and lipid metabolism. Not only does the liver control the storage, production, and secretion of glucose, it also produces and secretes very-low-density lipoproteins (VLDL) and takes up VLDL remnants, low-density lipoproteins (LDL), and albumin-bound fatty acids (FA). Insulin inhibits both hepatic glucose and VLDL-triglyceride (TG) production. It is not known, however, whether both processes are equally sensitive to insulin-mediated inhibition.

Hepatic glucose output (HGO) is determined by the rate of hepatic glycogen breakdown, which is regulated by glucose-6-phosphatase (G-6-Pase) and by the rate of hepatic gluconeogenesis, which is regulated by phosphoenolpyruvate carboxykinase (PEPCK). In the fed state, insulin inhibits HGO via inhibition of these two key regulatory enzymes (10, 19, 22). Insulin also stimulates glucose uptake by peripheral tissues, including skeletal muscle, heart, and adipose tissue. In these tissues, insulin stimulates translocation of glucose transporter-4 (GLUT4) mediating uptake of glucose (11). Previous studies have documented different dose-response effects of insulin on the HGO and peripheral glucose uptake (PGU). Rizza et al. (23) showed that HGO is more sensitive to inhibition by insulin than peripheral glucose uptake is to stimulation by insulin.

Hepatic VLDL-TG production is commonly assumed to be primarily a substrate-driven process (20), but insulin also plays an important role in the regulation of this VLDL-TG production. Insulin can inhibit the hepatic VLDL-TG production via direct and indirect mechanisms. The exact mechanism remains unclear, but it is thought that insulin can directly accelerate the degradation of apolipoprotein (apo)B, which is necessary for VLDL-TG secretion (2). An indirect effect of insulin is suggested to work via inhibition of hormone-sensitive lipase (HSL) in adipose tissue, leading to decreased plasma levels of FA and thus decreased flux of FA from the adipose tissue to the liver (16). However, in a study in humans, a metabolic relationship between insulin-mediated suppression of FA release from adipose tissue and FA flux to the liver on the one hand and the rate of hepatic VLDL-TG production on the other hand was not observed (6). A study by Lewis et al. (21) showed that, in normal individuals, the acute inhibition of VLDL-TG production by insulin in vivo was only partly due to the suppression of plasma FA and may also be due to an FA-independent process.

We investigated in wild-type C57Bl/6 mice whether HGO and hepatic VLDL-TG production are equally sensitive to insulin-mediated inhibition, using the hyperinsulinemic euglycemic clamp technique (7), which was adapted to mice as described previously by our group (26, 27). We found that the HGO is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production.

MATERIALS AND METHODS

Animals. For our experiments we used 12-wk-old male C57Bl/6 mice that were housed under standard conditions. The mice were fed a standard mouse/rat chow diet (Hope Farms, Woerden, The Netherlands) and water ad libitum. Mice were fasted for 2 h before the experiments and randomly assigned to respective groups, which were infused with different amounts of insulin. Per group, five to six animals were used. All animal experiments were approved by the Animal Ethics Committee from our institute.

Hyperinsulinemic euglycemic clamp. The clamp protocol was adapted from previously published studies performed by our group (26, 27). Food was withdrawn at 7 AM, and at 9 AM the mice were anesthetized with a combination of acetylpromazine (Vetranquil; Sanofi Santé Nutrition Animale, Libourne Cedex, France), midazolam (Dormicum; Roche, Woerden, The Netherlands), and fentanyl (Fen-
insula; Janssen-Cilag, Tilburg, The Netherlands). An infusion needle was placed into the tail vein, and basal glucose turnover rates were determined by infusion of D-[3-3H]glucose (0.6 μCi·kg⁻¹·min⁻¹; Amersham Biosciences, Little Chalfont, UK) alone for 45 min to achieve steady-state levels. After 45 and 60 min of infusion, blood samples (60 μl) were drawn from the tail into chilled capillary tubes (Hawksley and Sons, West Sussex, UK) coated with paraoxan (diethyl p-nitrophenyl phosphate; Sigma, St. Louis, MO) to prevent ex vivo lipolysis (29). These capillaries were kept on ice and spun for 5 min at 13,000 rpm to isolate the plasma, which was snap-frozen in liquid nitrogen and stored at −20°C until analysis.

After the basal period, a hyperinsulinemic clamp was started with the infusion of D-glucose solution in phosphate-buffered saline (PBS). A blood sample for glucose levels, exogenous glucose was infused via an adjustable infusion of 0.05 μmol·min⁻¹·kg⁻¹ body weight. The clamp experiments lasted 120 min from the slope of the curve and expressed as micromoles per hour per kilogram of body weight. Hyperinsulinemic HGO was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

**Calculations.** Under steady-state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance. The latter (in μmol·min⁻¹·kg⁻¹ body weight) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm·min⁻¹) divided by the plasma specific activity of [³H]glucose (dpm/μmol). The ratio was corrected for body weight. Hyperinsulinemic HGO was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

**Statistical analysis.** Results are presented as means ± SE for five animals per group. Differences between experimental groups were determined by the Mann-Whitney U-test. This means that groups were tested for linear trend (P<0.05) with increasing insulin levels. The level of statistical significance of the differences was set at P < 0.05. Analyses were performed using SPSS 12.0 for Windows software (SPSS, Chicago, IL) and Prism 4.0 (GraphPad).

### RESULTS

**Plasma glucose and insulin levels and glucose infusion rates during clamp analyses.** Plasma glucose levels during the basal and hyperinsulinemic clamp periods were not different between the groups (Table 1). At basal level, plasma insulin levels were not different between the groups, averaging 1.4 ng/ml. Under hyperinsulinemic conditions, steady-state plasma insulin concentrations in the respective groups averaged at 2.4, 3.6, 9.3, and 22.4 ng/ml with increasing insulin infusion rates. In addition, to maintain euglycemia during the respective insulin infusion rates, glucose infusion rate (GIR) increased concomitantly, as expected (P<0.01; Table 1).

**Dose-response effects of insulin on PGU and HGO.** We observed no differences in basal PGU between the groups (Table 2). During the hyperinsulinemic period, insulin dose-dependently stimulated PGU compared with the respective

### Table 1. Plasma levels of glucose and insulin and glucose infusion rates during hyperinsulinemic euglycemic clamp experiments

<table>
<thead>
<tr>
<th>Insulin Infusion, mU/h</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mM</th>
<th>Plasma Insulin, ng/ml</th>
<th>GIR, μmol·min⁻¹·kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Hyper</td>
<td>Basal</td>
<td>Hyper*</td>
</tr>
<tr>
<td>0</td>
<td>28.3 ± 0.9</td>
<td>7.5 ± 0.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3.5</td>
<td>25.9 ± 0.5</td>
<td>7.9 ± 0.6</td>
<td>10.1 ± 2.1</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>7</td>
<td>23.9 ± 0.7</td>
<td>8.9 ± 0.5</td>
<td>8.1 ± 1.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>14</td>
<td>26.0 ± 0.7</td>
<td>8.2 ± 0.3</td>
<td>8.2 ± 1.1</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>28</td>
<td>27.2 ± 1.4</td>
<td>8.1 ± 0.5</td>
<td>7.3 ± 0.9</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>*<em>Values represent means ± SE; n = 5–6 mice per group. Weight was measured at the beginning of the experiment. Plasma glucose and insulin levels were measured during basal and hyperinsulinic (Hyper) periods. GIR, glucose infusion rate necessary to maintain euglycemia during hyperinsulinemia. (†P &lt; 0.05 vs. basal group; <em>P&lt;0.01).</em></em></td>
<td></td>
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</table>

### Table 2. Effects of insulin infusion on PGU and HGO

<table>
<thead>
<tr>
<th>Insulin Infusion, mU/h</th>
<th>PGU, μmol·min⁻¹·kg⁻¹</th>
<th>HGO, μmol·min⁻¹·kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Hyper*</td>
</tr>
<tr>
<td>0</td>
<td>58.8 ± 9.4</td>
<td>NA</td>
</tr>
<tr>
<td>3.5</td>
<td>66.4 ± 6.3</td>
<td>57.2 ± 4.4</td>
</tr>
<tr>
<td>7</td>
<td>74.8 ± 11.1</td>
<td>79.0 ± 11.1</td>
</tr>
<tr>
<td>14</td>
<td>75.9 ± 5.3</td>
<td>152.0 ± 14.9†</td>
</tr>
<tr>
<td>28</td>
<td>61.4 ± 7.0</td>
<td>136.7 ± 17.0†</td>
</tr>
</tbody>
</table>

**Values represent means ± SE; n = 5–6 mice per group. During the clamp experiment, whole body glucose uptake (PGU) and hepatic glucose output (HGO) were measured under basal and hyperinsulinic conditions. †P < 0.05 vs. basal group; *P<0.01.**
basal levels ($P_{\text{trend}} < 0.01$). Similarly, basal HGO did not differ between the groups, whereas HGO was dose-dependently inhibited by insulin during the hyperinsulinemic conditions ($P_{\text{trend}} < 0.01$).

Dose-response effects of insulin on plasma FA levels and hepatic VLDL-TG production. The decrease in plasma FA levels was determined as a measure of insulin sensitivity of adipose tissue lipolysis. Upon infusion of insulin, plasma FA levels decreased dose dependently ($P_{\text{trend}} < 0.01$; Table 3). To measure the effect of insulin infusion on hepatic VLDL-TG production at the end of the hyperinsulinemic period, all groups of mice were injected with Triton WR-1339 to completely block plasma VLDL-TG lipolysis (1). Table 3 illustrates that insulin infusion leads to a dose-dependent decrease in hepatic VLDL-TG production ($P_{\text{trend}} < 0.01$; Table 3).

Comparison of PGU, plasma FA decrease, HGO, and hepatic VLDL-TG production regarding insulin sensitivity. Taken together, the data presented in Tables 2 and 3 clearly indicate that increasing plasma concentrations of insulin lead to a dose-dependent increase in PGU (Fig. 1A) and a dose-dependent decrease in plasma FA levels (Fig. 1B). Simultaneously, we measured in the same animals that, in the liver, the HGO (Fig. 1C) and hepatic VLDL-TG production (Fig. 1D) were inhibited dose dependently. For comparison of the dose-response characteristics of each of these effects of insulin, we estimated by extrapolation the insulin concentrations at which the half-maximal inhibitory or stimulatory effect was reached for these respective parameters (Table 4). It is obvious that, in the periphery, FA release from adipose tissue is more sensitive to plasma insulin than PGU. In the liver, HGO is more sensitive to plasma insulin levels than hepatic VLDL-TG production.

DISCUSSION

Insulin inhibits both hepatic glucose output and VLDL-TG production. Until now, it was not known whether both processes are equally sensitive to insulin-mediated inhibition. In the current study, we addressed this question and found that the HGO is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production.

Because in humans the liver is not readily accessible, mouse models are often used to investigate mechanisms of insulin resistance. The C57Bl/6 mouse is a model that is sensitive to diet-induced obesity and insulin resistance (24, 25). Therefore, we chose to use these mice for our studies of glucose and lipid metabolism. In general, there are three approaches to performing hyperinsulinemic clamp studies in mice in vivo. Some groups use free-moving mice with preimplanted catheters (13); other groups use awake but restrained mice (17); and some groups use anesthetized mice (26, 27). Each approach has its limitations. In free-moving mice, the effects of movement on the data of interest have to be taken into account; in restrained mice, the endocrine and neural effects of stress through re-

<table>
<thead>
<tr>
<th>Insulin Infusion, mU/h</th>
<th>FA, mM</th>
<th>%Basal*</th>
<th>HVP, μmol/min/kg⁻¹</th>
<th>%Basal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.69±0.06</td>
<td>100</td>
<td>159.6±9.0</td>
<td>100</td>
</tr>
<tr>
<td>3.5</td>
<td>0.63±0.05</td>
<td>91±9</td>
<td>189.5±19.5</td>
<td>119±12</td>
</tr>
<tr>
<td>7</td>
<td>0.37±0.05†</td>
<td>54±9†</td>
<td>158.1±17.6</td>
<td>99±11</td>
</tr>
<tr>
<td>14</td>
<td>0.24±0.03†</td>
<td>35±4†</td>
<td>90.3±6.0†</td>
<td>57±4†</td>
</tr>
<tr>
<td>28</td>
<td>0.25±0.02†</td>
<td>36±3†</td>
<td>83.3±7.7†</td>
<td>52±5†</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 5–6 mice per group. After the clamp experiment, plasma fatty acid (FA) levels and hepatic VLDL-triglyceride production (HVP) rate were measured under basal and hyperinsulinemic conditions. †P < 0.05 vs. basal group; * $P_{\text{trend}} < 0.01$. Fig. 1. Hepatic glucose production is more sensitive to insulin-mediated inhibition than hepatic very-low-density lipoprotein-triglyceride (VLDL-TG) production. During a hyperinsulinemic euglycemic clamp experiment with different plasma insulin concentrations per group, we measured the stimulation of peripheral glucose uptake (A) and the decrease in plasma fatty acids (FA; B). Simultaneously, we measured insulin-mediated inhibition of hepatic glucose production (C) and of hepatic VLDL-TG production (D). Dotted lines indicate maximal and half-maximal effects of insulin.
The half-maximal effect of insulin was determined for each parameter during hyperinsulinemic clamp studies. We estimated the half-maximal effect by extrapolation from the curves using the numbers presented in Tables 2 and 3.

Table 4. Plasma insulin levels at half-maximal effect

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plasma Insulin Level, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>3.0</td>
</tr>
<tr>
<td>HGO</td>
<td>3.6</td>
</tr>
<tr>
<td>PGU</td>
<td>6.4</td>
</tr>
<tr>
<td>HVP</td>
<td>6.8</td>
</tr>
</tbody>
</table>

By using this animal model, we were able to measure the effect of insulin on HGO and hepatic VLDL-TG production, and at the same time also on PGU and plasma FA levels, the latter as a measure of insulin sensitivity of adipose tissue lipolysis. Although an exact extrapolation for determination of half-maximal effect could not be made, Fig. 1 shows that plasma FA levels, PGU, HGO, and hepatic VLDL-TG production differ in insulin sensitivity. By comparison of the insulin levels at the half-maximal effect, we observed that HGO is more sensitive to insulin-mediated regulation than PGU. This is in concordance with the study of Rizza et al. (23), who showed in humans that half-maximal suppression of HGO occurs at insulin levels of 29 μU/ml (~0.9 ng/ml), whereas half-maximal stimulation of PGU occurs at 55 μU/ml (~1.8 ng/ml). Furthermore, the suppression of plasma FA appears to be much more sensitive to insulin than the stimulation of PGU. In fact, adipose tissue lipolysis and PGU are two completely different processes. Although lipolysis by HSL takes place in adipose tissue only, insulin-stimulated PGU occurs both in adipose tissue and in muscle. Therefore, it is not possible to quantitatively compare these peripheral parameters regarding their regulation by insulin under these conditions.

The observation that hepatic VLDL-TG production is much less sensitive to the inhibitory effect of insulin than HGO suggests that these two processes are regulated differently. In the regulation of HGO, insulin inhibits the forkhead box Other-1 (FoxO1), which binds to promoter regions of genes encoding the enzymes G-6-Pase and PEPCK (4), which are important regulators of glycolysis and gluconeogenesis, respectively (10, 19, 22). The molecular mechanism underlying the insulin-mediated suppression of hepatic VLDL-TG production is not completely clear. Studies have shown that insulin can inhibit the lipiddation of pre-VLDL via inhibition of microsomal TG transfer protein (MTTP) (15, 28). MTTP is the enzyme that catalyzes the fusion of the pre-VLDL with a lipid droplet, thereby rendering the pre-VLDL into a mature VLDL particle ready for secretion. In addition, in vitro studies have shown that insulin stimulates the degradation of apoB in hepatocytes (3, 5, 9). Decreased intracellular apoB availability leads to a decreased hepatic VLDL-TG production. Furthermore, insulin is known to inhibit HSL in adipose tissue, leading to decreased plasma levels of FA and thus to decreased flux of FA from adipose tissue to the liver, which will eventually decrease FA reesterification into TG in hepatocytes (16). It has indeed been shown, that in the presence of hyperinsulinemia the liver secretes less and smaller VLDL particles (12). However, in a study in humans, an association between insulin-mediated suppression of FA release from adipose tissue and FA flux to the liver on the one hand, and the rate of hepatic VLDL-TG production (estimated from the monoexponential slope of VLDL-TG [3H]glycerol enrichment) on the other hand, was not observed (6). Another semiquantitative study in humans also showed that, in normal individuals, the acute inhibition of VLDL-TG production by insulin in vivo is only partly due to the suppression of plasma FA (21). Accordingly, in the current study we could not find a significant correlation between a decrease in plasma FA levels and a decrease in hepatic VLDL-TG production during hyperinsulinemia. Apparently, plasma FA levels and FA availability to the liver per se do not determine hepatic VLDL-TG production. In accord with this notion, we have previously shown that acute redirection of hepatic FA flux from β-oxidation to storage does not affect hepatic VLDL-TG production (8). We suggest that, under the conditions of our experiment, insulin exerts direct effects on hepatic VLDL-TG production that are apparently of greater importance than the indirect effects via suppression of FA release from adipose tissue or FA availability in general, at least under the conditions of our experiments. We hypothesize that hepatic VLDL-TG production is inhibited by insulin via a combination of the three different mechanisms described above and may therefore be less sensitive to insulin.

Metabolic zonation may also be a factor involved in the difference in insulin sensitivity of HGO vs. hepatic VLDL-TG production. Hepatic metabolic pathways are not uniformly distributed across the liver (14). Within the liver acinus, different zones exist. In the efferent perivenous zone, more FA synthesis takes place, and the activity of acetyl-CoA carboxylase is much higher than in the afferent periportal area. The perivenous zone also has a larger capacity to reesterify exogenous FA into TG. Carbohydrate metabolism also differs between the two areas. Glucose uptake for glycogen synthesis occurs mainly in the perivenous zone, whereas the generation of glucose via glycogenolysis and gluconeogenesis occurs mainly periportal. Furthermore, although insulin receptor mRNA is homogenously distributed in the liver acinus, insulin receptor protein is expressed mainly in the perivenous area in rat liver (18). How these differences in metabolic zonation may be reflected in differential regulation of HGO and hepatic VLDL-TG production by insulin is subject to speculation.
In summary, our study shows that HGO is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production. This is of major importance for the use of the gold standard of measuring insulin sensitivity, the hyperinsulinemic euglycemic clamp technique. A low insulin dose already suppresses HGO, whereas no effect on hepatic VLDL-TG production may be observed. Infusion of high insulin dosages may lead to the overlooking of subtle differences in hepatic insulin sensitivity, especially with regard to the HGO.

GRANTS
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REFERENCES