Preserved direct hepatic insulin action in rats with diet-induced hepatic steatosis

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Buettner, Roland, Iris Ottinger, Jürgen Schölmérich, and L. Cornelius Bollheimer. Preserved direct hepatic insulin action in rats with diet-induced hepatic steatosis. Am J Physiol Endocrinol Metab 286: E828–E833, 2004. First published January 13, 2004; 10.1152/ajpendo.00453.2003.—Recent in vivo studies have demonstrated a strong negative correlation between liver triglyceride content and hepatic insulin sensitivity, but a causal relationship remains to be established. We therefore have examined parameters of direct hepatic insulin action on isolated steatotic livers from high-fat (HF)-fed rats compared with standard chow (SC)-fed controls. Direct hepatic action of insulin was assayed in Wistar rats after 6 wk of HF diet by measuring the insulin-induced suppression of epinephrine-induced hepatic glucose output in an isolated liver perfusion system. Insulin-induced activation of glycogen synthase was measured by quantifying the incorporation of radioactive UDP-glucose into glycogen in HF and SC liver lysates. HF diet induced visceral obesity, mild insulin resistance, and hepatic steatosis. Both suppression of epinephrine-induced glycogenolysis and activation of glycogen synthase by insulin were sustained in HF rats; no significant difference from SC controls could be detected. In conclusion, in our model, triglyceride accumulation into the liver was not sufficient to impair direct hepatic insulin action. The data argue for an important role of systemic factors in the regulation of hepatic glucose output and hepatic insulin sensitivity in vivo.

insulin resistance; high-fat diet; liver perfusion

AN ELEVATED HEPATIC GLUCOSE OUTPUT (HGO), even under hyperglycemic conditions, is a major feature of obesity-linked type 2 diabetes. This impairment of hepatic glucose metabolism is classically thought to derive from hepatic insulin resistance, meaning an insufficient ability of insulin to suppress HGO in prediabetic and diabetic patients as well as in rodent models of type 2 diabetes (24, 28, 29). In recent years, much progress has been made in understanding insulin receptor signal transduction and intracellular pathways mediating insulin action downstream of the receptor in insulin-dependent tissues, such as muscle, fat, and liver (40). Circulating factors such as free fatty acids or adipose tissue secretory products have been postulated as humoral modulators of tissue insulin sensitivity (see recent review in Ref. 14). Recent studies link intracellular triglyceride and/or fatty acid accumulation, a common feature of diet-induced obesity, with insulin resistance in the muscle (7, 18), as well as with perturbed insulin secretion in fat-laden pancreatic islets (33, 37).

Clinical experience has associated insulin-resistant states, such as the metabolic syndrome, with fat accumulation in the liver over a long period of time (21). In patients with hepatic steatosis, but without known diabetes, insulin resistance as measured by homeostasis models is a common trait (8); impaired glucose tolerance as a hallmark of insulin resistance can be expected in ≤30% of such patients (31, 38). Clamp experiments in humans reveal an impaired suppression of HGO by insulin in patients with hepatic steatosis before the onset of impaired glucose tolerance or overt type 2 diabetes (32). Animal studies have similarly described negative correlations between perturbed hepatic insulin action and liver fat content (19).

Although these data clearly show that hepatic steatosis is commonly associated with obesity, insulin resistance, and a decreased insulin-induced suppression of HGO, they do not prove a causal relationship between hepatic fat accumulation and impaired insulin action on the liver. After all, the latter could also result indirectly from systemic factors influencing the liver’s insulin sensitivity in vivo. To our knowledge, studies proving a defect in insulin action on isolated fatty livers without single genetic defects of glucose or lipid metabolism, which would allow us to define the role of direct insulin action on steatotic livers in insulin-resistant states, do not exist at present.

This issue obviously cannot be studied in humans. We therefore used the Wistar rat high-fat-diet model of hepatic steatosis and insulin resistance (7) to quantify direct hepatic insulin action in fatty livers. For this, the effects of insulin on HGO and on the insulin-dependent activation of hepatic glycogen synthase were analyzed using a liver perfusion system. Our results show sustained hepatic insulin action in high-fat-fed animals despite a sevenfold elevation of hepatic triglyceride content; they therefore argue against a simple equation of hepatic steatosis with direct hepatic insulin resistance.

MATERIALS AND METHODS

Experimental animals. Male Wistar rats were purchased from Charles River (Sulzfeld, Germany) at a weight of 150 g. After 3 days of acclimatization, rats were caged with free access to water and to either a high-fat (HF) diet (43% of calories from fat) or standard rat chow diet (11% of calories from fat). Animals were held on a 12:12-h light-dark cycle. All animal procedures were approved by the local animal rights committee and complied with the German Law on Animal Protection as well as the National Research Council’s Guide for the Care and Use of Laboratory Animals, 1996.

Experimental design. Obesity and insulin resistance were induced in male Wistar rats by a 6-wk HF diet; controls were fed with standard chow (SC). The model was characterized metabolically by measuring basal fasting plasma insulin, glucose, triglyceride, and free fatty acids, and intraperitoneal insulin tolerance tests were performed. Retropitoneal, epididymal, and perirenal fat pads were removed and

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weighed, and liver triglyceride content was measured. Fed animals were used for liver perfusion experiments to analyze the hepatic glucose output with or without exposure to epinephrine and insulin or the insulin stimulation of glycogen synthase activity, as we will describe in detail. Unless otherwise stated, all reagents were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany) at the highest purity grade available.

**HF diet.** A premixed HF diet (no. 1571) was purchased from Altromin (Lage, Germany). In terms of percentage by weight, it consisted of 20% fat (mainly lard), 42% carbohydrate, and 21% protein.

**Liver perfusion experiments.** Krebs-Ringer-Henseleit (KRB) buffer (7) containing 4 mmol/l glucose and 2 mmol/l lactate and oxygenated by continuous gassing with carbogen (95% O₂–5% CO₂) at 37°C, and a pH of 7.4 with additions as will be described) was used as perfusate. The experiments were performed between 8:00 and 10:00 AM with fed HF or SC rats anesthetized with pentobarbital sodium (50 mg/kg body wt). Single-pass liver perfusion was then performed. For this, a 16-gauge catheter was inserted into the portal vein and secured by a ligature, and perfusion was started at a flow rate of 3–4 ml/min−1 g liver−1. To avoid an elevation of portal venous pressure, the vena cava inferior was immediately cut after the start of perfusion. A 23-gauge catheter was inserted into the common bile duct. After a stable perfusate flow was established with an adequate liver perfusion, assayed by homogenous color change and liver temperature as well as a portal venous pressure <5 cmH₂O, the vena cava inferior was ligated, and the equilibration phase started. Glucose output was measured every 5 min with a portable glucose monitor (Roche Diagnostics, Mannheim, Germany). After 30 min, when a stable glucose output had been established, an epinephrine infusion (50 nmol/l) was started by use of the equilibration phase started. Glucose output was measured by means of the GPO-triglyceride kit (Sigma) with appropriate biochemical parameters, are shown in Table 1. On average, HF rats were 14% heavier than SC controls (P < 0.01), demonstrating that the HF diet induced visceral obesity. Liver triglyceride content was sevenfold higher in HF rats (P = 0.01). HF rat insulin levels were 35% elevated over those of SC controls, and fasting plasma glucose was ~20% higher in HF-fed rats (P ≤ 0.05). These results indicated insulin resistance in HF animals. This was confirmed by performing insulin tolerance tests. Here, tail whole blood glucose levels in SC rats dropped from 5.3 ± 0.3 mmol/l to a minimal 3.8 ± 0.2 mmol/l at 35 min after intraperitoneal insulin injection, whereas HF rats injected with an equal amount of insulin per kilogram body weight showed no clear decrease in glucose levels at any time.
point. Consequently, insulin-induced glucose disposal estimated from the AUC for glucose concentration after 60 min was ~25% in SC rats, whereas HF rats showed no significant change from controls (P < 0.05 compared with SC).

Effects of HF diet on basal and epinephrine-stimulated hepatic glucose output. Livers of fed HF and SC rats were perfused with KRH buffer containing 4 mmol/l glucose and 2 mmol/l lactate as gluconeogenic substrate. The time course of the HGO with and without epinephrine stimulation is depicted in Figs. 1A (SC) and 2A (HF). SC and HF rats showed similar basal HGO values of 1.34 ± 0.1 and 1.42 ± 0.05 μmol-g liver⁻¹ min⁻¹. After addition of epinephrine at 50 nmol/l, we observed a rapid increase in HGO of 2.5-fold over basal conditions in SC rats. This effect did not differ significantly in HF rats, with a 2.8-fold increase of HGO over basal. In both groups, epinephrine-stimulated HGO was continuously elevated for ~16 min. It then declined very slowly but was still significantly elevated over basal after ~30 min of epinephrine infusion.

Effects of HF diet on insulin-induced suppression of HGO. In the next set of experiments, the liver perfusions were performed as described above, with an additional continuous insulin infusion from the start to the end of perfusion (Figs. 1B and 2B). We did not observe any relevant effects of insulin on basal HGO in SC or HF rats. During the first 10 min of epinephrine infusion, HGO increased rapidly to 2.2-fold and 2.7-fold over basal in SC and HF rats, respectively. This initial HGO increment was slightly, but not significantly, lower than that measured in the previous experiments without insulin for both SC and HF rats. After time point t = 10 min, HGO decreased steadily in the insulin-exposed livers, leading to a near basal hepatic glucose release at 20 min after the start of epinephrine. This insulin-induced suppression of epinephrine-stimulated HGO was quantified by subtracting the total glucose output (calculated by AUC analysis) over basal HGO between the epinephrine-treated and the epinephrine and/or insulin-treated groups. When SC were compared with HF rats with respect to this parameter, no significant difference was found (33 ± 10% in SC rats, 32 ± 10% in HF rats, respectively, P = 0.96).

Activation of GS by insulin in HF and SC rats. To further analyze the direct hepatic insulin sensitivity in SC and HF rats, we measured the activation of GS in livers perfused with insulin at 100 μmol/l. The results are shown in Fig. 3. Both basal and insulin-stimulated GSα activities tended to be higher in HF rats, whereas the relative increment in enzyme activity induced by insulin was similar in the two diet groups (fold increase for SC: 1.68 ± 0.11; for HF: 1.60 ± 0.08, P = 0.65, when diet groups were compared with each other).

**DISCUSSION**

Liver insulin resistance develops in patients and rodent models with elevated intrahepatic triglycerides (hTG) (2, 8, 19). It is also a common trait in patients with type 2 diabetes, who often present with hepatic steatosis (31). Although the association between hepatic insulin resistance and elevated liver triglycerides demonstrated by these and other studies seems obvious, the direct effects of insulin on fat-laden livers have not been well characterized. Therefore, the goal of this study was to analyze parameters of hepatic insulin action in steatotic livers of HF rats compared with SC-fed controls.

By using a lard-based high-fat rodent diet, we were able to induce visceral obesity and moderate signs of whole body insulin resistance in normal male Wistar rats. The animals showed a marked rise in hTG content, as previously described (30). Hepatic insulin action was assessed in HF-fed and control rats by using an isolated liver perfusion system that enabled us to look at direct insulin effects. By this method, the multiple confounding factors influencing insulin action in vivo clamp.

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**Table 1. General and metabolic plasma characteristics of rats fed SC or HF diet for 6 wk**

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>HF</th>
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<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
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</tr>
<tr>
<td>Body wt, g</td>
<td>390 ± 3</td>
<td>457 ± 7*</td>
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<tr>
<td>Visceral fat pads</td>
<td></td>
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<tr>
<td>g</td>
<td>8.8 ± 1.1</td>
<td>15.9 ± 0.8*</td>
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<tr>
<td>% body wt</td>
<td>2.2 ± 0.3</td>
<td>3.4 ± 0.2*</td>
</tr>
<tr>
<td>Liver wt</td>
<td></td>
<td></td>
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<tr>
<td>g</td>
<td>12.4 ± 0.2</td>
<td>14.7 ± 0.2*</td>
</tr>
<tr>
<td>% body wt</td>
<td>3.2 ± 0.05</td>
<td>3.2 ± 0.05</td>
</tr>
<tr>
<td>Liver triglyceride μg/ mg liver</td>
<td>1.6 ± 0.4</td>
<td>10.4 ± 2.5*</td>
</tr>
<tr>
<td><strong>Metabolic plasma</strong></td>
<td></td>
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<tr>
<td>Glucose, mmol/l</td>
<td>4.1 ± 0.2</td>
<td>4.9 ± 0.3*</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>174 ± 7</td>
<td>241 ± 21</td>
</tr>
<tr>
<td>Free fatty acids, μmol/l</td>
<td>0.33 ± 0.01</td>
<td>0.41 ± 0.01*</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>0.50 ± 0.03</td>
<td>0.66 ± 0.04*</td>
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Values are means ± SE of 5 animals. SC, standard chow; HF, high-fat diet. *P < 0.05.
settings, in which systemic insulin effects on glucose as well as on lipid and amino acid metabolism may influence hepatic glycolysis and gluconeogenesis, could be ruled out. Hepatocyte cultures were not employed, because the time-consuming isolation procedure might have led to changes of HTG deposits and also might have influenced the putative insulin-resistant phenotype. We used a cell-free perfusate to avoid changes in lactate concentration originating from erythrocyte metabolism. The perfusions were performed with fed rats to ensure sufficient glycogen deposits. A physiological perfusate glucose concentration was chosen because hyperglycemia is known to attenuate diet-induced insulin resistance (10), whereas low glucose conditions would have increased hepatic glucose production, thereby diminishing glycogen stores and potentially lowering the HGO-suppressing effect of insulin. Relevant hypoxia during the perfusions, which would influence the results, can be ruled out, because the parameters of liver vitality, in particular oxygen extraction, were stable during the perfusions.

Basal HGO, as assessed by the portocaval glucose difference during liver perfusions, did not differ significantly between HF rats and SC controls. These results argue against an increase in basal hepatic glucose production induced solely by chronic hTG elevation, although we cannot rule out that a putative HGO elevation might have been counteracted by concomitantly decreased hepatic glycogen stores, as observed previously in high-fat-fed rats (23). Insulin at a low physiological portal concentration was not able to suppress basal HGO in either experimental group. This is in keeping with previous results, demonstrating that insulin by itself does not change HGO in isolated liver perfusion systems at normal glucose concentrations (41).

The glycolytic catecholamine epinephrine effectively stimulated HGO; the extent and duration of epinephrine-induced HGO stimulation were markedly lowered by insulin in SC rats. These results correspond with earlier work demonstrating a strong reduction of adrenergically stimulated glycolysis by insulin in comparable perfusion systems (11, 16, 22). In livers of HF rats, insulin also effectively decreased epinephrine-induced HGO stimulation. This indicates that insulin is able to directly suppress adrenergically stimulated hepatic glycolysis regardless of liver triglyceride content. To our knowledge, no previous study has measured direct hepatic insulin action in high-fat-fed rats with defined hepatic steatosis by use of an isolated perfusion system. Ikeda and Fujiyama (17) showed insulin resistance in perfused livers of high-fructose-fed rats, but they did not state hepatic triglyceride content, making our results difficult to compare. To confirm the sustained action of insulin in fatty livers with a second experimental parameter, we measured GS activity in livers perfused with and without insulin. We observed clear insulin-induced increments of GS activity, showing that the experimental system was valid for analysis of hepatic insulin action. No clear difference between HF and SC rat livers was found (Fig. 3). Again, to our knowledge, no comparable previous results exist.

In conclusion, no signs of resistance to direct actions of insulin were detected in steatotic rat livers when two different parameters of hepatic insulin action were observed. These results do not support the simple preconception that elevated liver triglyceride deposition by itself necessarily impairs hepatic insulin action, e.g., by influencing binding of insulin to its receptor, insulin receptor activity, or postreceptor signal transduction. This notion is supported by a previous study, which failed to demonstrate clear deteriorating effects of hepatic steatosis when insulin-induced activation of phosphatidylinos...
sitot 3-kinase in livers of high-fat-fed rats was analyzed (3). However, other authors have reported a decreased insulin receptor kinase activity (39), so this issue must remain controversial. Also, when interpreting our results, it should be considered that the results we obtained apply only to the insulin suppression of hepatic glycogenolysis. HGO deriving from gluconeogenesis might be regulated differently, and direct insulin action on this part of hepatic glucose metabolism remains to be quantified in fat-laden livers.

Substantial evidence for an impairment of direct insulin action on fatty livers does exist from in vivo studies. Seppala-Lindroos et al. (32) recently demonstrated that basal HGO remains unchanged in subjects with hepatic steatosis despite elevated fasting insulin levels. Basal HGO is regulated by the portal vein insulin concentration in vivo (35). Taken together, these studies suggest that higher insulin concentrations were needed to restrain HGO in these subjects. The fact that steatotic livers show an impairment of insulin action in vivo, but not in our isolated perfusion system, argues for the hypothesis of a secondary origin of hepatic insulin resistance. Extrahepatic factors that regulate aspects of hepatic insulin action, e.g., suppression of HGO, would then be pathologically changed in insulin-resistant states. Previous authors examining hepatic insulin action have come to similar conclusions. The “single gateway theory” proposed by Bergman (4) states that insulin resistance of the adipocyte impairs insulin-induced suppression of lipolysis. This leads to an elevation of free fatty acids, which in turn stimulate hepatic glucose production. Key assumptions of this theory have been confirmed during the last few years (1, 20, 25, 34), but they are challenged by recent findings in liver-specific insulin receptor knockout mice, demonstrating that both the direct and the indirect insulin actions on HGO require an intact insulin-signaling pathway in the liver (12).

Other studies following the single gateway theory have suggested alternative extrahepatic regulators of HGO. New studies show that adipocyte-derived hormones, such as resistin, decrease the hepatic insulin effect (27). Also, it has recently been demonstrated that mice lacking insulin receptors in the central nervous system develop insulin resistance (6), and central insulin antagonism impairs the ability of circulating insulin to inhibit hepatic glucose production (26). Other arguments against hepatic triglyceride content as the main regulator of the insulin effect on the liver come from rodent studies that use different interventions to increase hepatic insulin action. Insulin-induced HGO suppression was reversed to normal after a 3-day diet change, which cannot reasonably be expected to reverse hepatic steatosis, in HF-fed rats (15). Removal of visceral fat in obese rats markedly improved hepatic insulin action over that in controls (13). In summary, a multitude of recent results strongly argue for the existence of an extrahepatic regulation of hepatic insulin action, which might be altered in insulin-resistant and diabetic states.

When our findings are taken together, we demonstrate sustained direct hepatic insulin action in steatotic livers of HF-fed rats in terms of suppression of glycogenolysis. Elevated hTG, therefore, do not necessarily interfere with insulin receptor function or intracellular insulin signaling. The hepatic “insulin resistance” observed in vivo may at least partially result indirectly, i.e., be caused by circulating factors that interfere with insulin signaling and/or hepatic glucose production.

REFERENCES


