Intrahepatic Mechanisms Underlying the Effect of Metformin in Decreasing Basal Glucose Production in Rats Fed a High-Fat Diet

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The aim of this study was to understand by which intrahepatic mechanism metformin (Met) may inhibit basal hepatic glucose production (HGP) in type 2 diabetes. We studied rats that were fed for 6 weeks a high-fat (HF) diet, supplemented (HF-Met) or not (HF) with Met ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$). Basal HGP, assessed by 3-[^{3}H]glucose tracer dilution, was lower by 20% in HF-Met rats compared with HF-rats: 41.6 ± 0.7 vs. 52 ± 1.5 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (means $\pm$ SE, $n = 5$; $P < 0.01$). Glucose-6 phosphatase (Glc6Pase) activity, assayed in a liver lobe freeze-clamped in situ, was lower by 25% in HF-Met rats compared with HF-rats (7.9 ± 0.4 vs. 10.3 ± 0.9 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ wet liver; $P < 0.05$). Glucose-6 phosphate and glycogen contents, e.g., 42 ± 5 nmol/g and 3.9 ± 2.4 mg/g, respectively, in HF-rats were dramatically increased by three to five times in HF-Met rats, e.g., 118 ± 12 nmol/g and 19.6 ± 4.6 mg/g ($P < 0.05$ and $P < 0.01$, respectively). Glucose-6 phosphate dehydrogenase activity was increased in HF-Met compared with HF rats (1.51 ± 0.1 vs. 1.06 ± 0.08 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$; $P < 0.01$). Intrahepatic lactate concentration tended to be lower in the Met-group (-30%; NS), whereas plasma lactate concentration was higher in HF-Met rats (1.59 ± 0.15 mmol/l) than in HF rats (1.06 ± 0.06 mmol/l; $P < 0.05$). We concluded that Met decreases HGP in insulin-resistant HF-fed rats mainly by an inhibition of hepatic Glc6Pase activity, promoting glycogen sparing. Additional mechanisms might involve the diversion of glucose-6 phosphate into the pentose phosphate pathway and an inhibition of hepatic lactate uptake. *Diabetes* 51:139–143, 2002

Metformin (Met) has been used as an oral pharmacological agent in the treatment of patients with type 2 diabetes for the past 40 years (1). Recently, it has been strongly suggested that its primary action is in the improvement of insulin sensitivity of the liver, resulting in a decrease in basal endogenous glucose production (2–5). The hepatic mechanism of action of Met is not yet fully elucidated. For example, whether gluconeogenesis (3) or glycogenolysis (5) is the main pathway altered by Met is not clear. The knowledge of such a mechanism is a crucial issue as it may uncover important new target steps in the treatment of type 2 diabetes. In a preceding work devoted to the effect of Met in insulin-resistant rats fed a high-fat (HF) diet, we suggested that the restoring effect of Met on the liver insulin sensitivity might involve an inhibition of the glucose-6 phosphatase (Glc6Pase) activity (6). More recently, we showed that reliable conditions to characterize biochemically the main determinants of hepatic glucose production (HGP) under their in situ metabolically active form, e.g., Glc6Pase, glucokinase (GK), and the glucose-6 phosphate concentration (Glc6P), were achieved by freeze-clamping a liver lobe at $-196°C$ in anesthetized rats (7). In the work presented herein, we used the latter approach to get a better understanding of the intrahepatic biochemical mechanisms of action of Met on basal HGP under conditions of insulin resistance.

RESEARCH DESIGN AND METHODS

Animals and diets. Male Sprague-Dawley rats (IFFA-CREDO, Lyon, France) were used in this study. Control rats were fed a standard laboratory diet (50% starch/glucose, 23.5% proteins, 12% water, 5.5% mineral salts, 4% cellulose; weight basis, Unité d'Alimentation rationnelle [UAR], Epinay-sur-Orge, France). HF-rats were fed a lipid-enriched diet (24% starch/glucose, 25% casein, 36% carthame oil, 6% cellulose, 7% mineral salts, 1% vitamins, 1% choline; weight basis, UAR) given ad libitum for 6 weeks. HF-rats treated with Met (HF-Met) were given the drug ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) mixed with the diet for the last week (6). Rats were randomized regarding to Met/no Met. No placebo was added to the diet in the untreated group. Rats had free access to water. Food was withdrawn 5 h before the experiments. Apart from this latter fasting period, rats were not fasted when in their light cycle.

Protocols for the determination of HGP. HF and HF-Met rats were anesthetized using pentobarbital (one injection at a dose of 7 mg/100 g body wt). Polyethylene catheters were placed in the left carotid artery for blood sampling and in the right jugular vein for infusion of 3-[^{3}H]glucose (0.24 μCi/min; Isotopchim, Ganagobie, France) diluted in saline, for 3 h. The labeled glucose infusion was primed (2.4 μCi/min) for 1 min. Glycemia was monitored every 15 min using a Glucomatic Esprit (Bayer Diagnostics, Puteaux, France). Body temperature was maintained at 37.5°C using a heating blanket monitored by a rectal probe. A laparotomy was performed 15 min before the final blood sampling (180 min). The incision was protected using wet gauze (with saline). Just after the final blood sampling, a liver lobe was frozen in situ at $-196°C$ using tongs precooled in liquid nitrogen and then stored at $-80°C$ (7). Basal HGP was calculated from the specific activity of plasma 3-[^{3}H]glucose at 180 min as reported previously (6,7). Preliminary experiments involving blood samplings every 10 min from 0 to 60 min and at 120 and 180 min indicated that a steady-state specific activity of plasma 3-[^{3}H]glucose was always reached from 30 min and maintained up to 180 min under these conditions, ascertaining the validity of HGP determination at the end of infusions (not shown). For checking insulin resistance in both groups of HF rats, HGP was also determined under conditions of insulin infusion at 480 pmol/h, with infusion

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TABLE 1
Effect of Met on body weight and plasma glycemic and lipidic parameters in HF rats

Diet/treatment	Body weight (g)	Insulin (pmol/l)	Glucagon (ng/l)	Glucose (mmol/l)	Lactate (mmol/l)	FFA (mmol/l)	TG (g/l)
HF	348 ± 12	278 ± 58	279 ± 38	8.1 ± 0.2	1.06 ± 0.06	0.33 ± 0.01	0.47 ± 0.05
HF-Met	340 ± 10	234 ± 24	306 ± 90	8.2 ± 0.3	1.59 ± 0.15*	0.39 ± 0.03	0.52 ± 0.04

Data are means ± SE ($n = 5$), different from HF, *$p < 0.05$.

of glucose (1.67 mol/l) at an adapted rate to maintain euglycemia, in comparison with a control group fed a standard diet, as described in detail in our previous reports (6,7).
Enzyme and metabolite assays. A frozen liver sample was powdered at liquid N_2 temperature and homogenized in 10 mmol/l HEPES, 250 mmol/l sucrose (pH 7.4; 9 vol/g wet tissue) by sonication (8). Specific Glc6Pase activity was assayed as previously described, using a method involving β-glycerophosphatase activity determinations to clear nonspecific phosphatase activities (7,8). GK was assayed in 12,000g supernatants of liver homogenates as described by Bontemps et al. (9). Glucose-6 phosphate dehydrogenase (Glc6PDH) was assayed according to Löhr and Wahler (10). Glycogen phosphorylase a (GPa) and glycogen synthase (GSa) were assayed as described by Hue et al. (11). Glycogen and Glc6P were assayed according to Keppler and Decker (12) and Lang and Michal (13), respectively. Liver lipids were extracted according to Bligh and Dyer (14). Triglycerides (TG) were separated by thin-layer chromatography on chromarods SIII (15) and analyzed by flame ionization using a Iatroscan TH-10 (Iatron Laboratories, Tokyo, Japan). Quantification was performed from comparisons with internal standards and calibration curves of purified TG (Sigma, La Verpillière, France).
Other methods and calculations. Plasma insulin and glucagon were determined by radioimmunoassay (16,17). Plasma glucose and lactate were determined as described (18,19). Plasma free fatty acids (FFA) and TG were determined using commercial kits (Wako, Dardilly, France, and Sigma, respectively). Protein was determined using Lowry's method (20). Procedures for the determination of the amount of immunoreactive Glc6Pase protein were as described in detail previously (7,21). Statistical analyses were performed using Fisher's exact test (22).

RESULTS

To check that HF rats exhibited altered insulin sensitivity of endogenous glucose production, we first studied the suppression of HGP by insulin in both HF groups in comparison with a control (starch-fed) group. In control rats, HGP (76 ± 7 μmol · kg^{-1} · min^{-1} in the basal state, mean ± SE, $n = 5$) was markedly inhibited (by ~75%) upon insulin infusion under conditions of euglycemia (21 ± 10 μmol · kg^{-1} · min^{-1}; $P < 0.01$), in agreement with our previous data (6,7). Basal HGP was lower in HF-Met rats than in HF rats (41.6 ± 1.7 vs. 52 ± 1.5 μmol · kg^{-1} · min^{-1}, respectively, $n = 5$; $P < 0.05$). HGP was suppressed to very similar levels in both groups (31 ± 9 and 30 ± 2 μmol · kg^{-1} · min^{-1} in HF and HF-Met rats, respectively;

$P < 0.05$ and $P < 0.01$ vs. respective basal HGP). This represented <40% inhibition by insulin in both groups. Final plasma insulin levels were 449 ± 54, 825 ± 25, and 742 ± 46 pmol/l in control, HF, and HF-Met rats, respectively ($n = 5$; "control" value different from HF and HF-Met values; $P < 0.05$). Because the effect of insulin infusion on the key enzyme activities and metabolites involved in HGP in control rats and in HF and HF-Met rats had been the matter of our previous works (6,7), we further focused the study herein on the intrahepatic biochemical mechanism of action of Met in the basal state in HF-fed rats, a situation in which the effect of the drug was obvious.

HF rats were identical on the basis of body weight and of plasma glucose, FFA, TG, insulin, and glucagon, whether Met had been added to the diet or not. In contrast, there was a low but significant increase in the plasma lactate concentration in the Met-treated group (Table 1).

Glc6Pase activity assayed in the homogenates from freeze-clamped liver lobes was lower (by ~25%) in HF-Met rats than in HF rats (7.9 ± 0.4 vs. 10.3 ± 0.9 μmol · kg^{-1} · min^{-1} wet liver, respectively; $P < 0.05$) (Fig. 1). However, no difference could be detected between both groups in regard to the amount of immunoreactive Glc6Pase protein studied by Western blot (Fig. 2). Glc6Pase activity was the same in control starch-fed rats (8.1 ± 0.6 μmol · kg^{-1} · min^{-1}; not shown) and in HF-Met rats (see above). There was no significant difference between the two HF groups regarding the liver GK activity (~0.6 μmol · min^{-1} · g^{-1}). In contrast, the GK activity was higher ($P < 0.01$ versus both HF groups) in control rats (1.11 ± 0.03 μmol · min^{-1} · g^{-1} liver; not shown). The Glc6P content was very low in HF rats (42 ± 9 nmol/g wet liver) and was three- to fourfold higher in HF-Met rats (118 ± 12 nmol/g; $P < 0.05$) (Fig. 1). The Glc6P content was higher in control rats (170 ± 18 nmol/g; $P < 0.01$ and $P < 0.05$ versus HF-Met and HF rats, respectively).

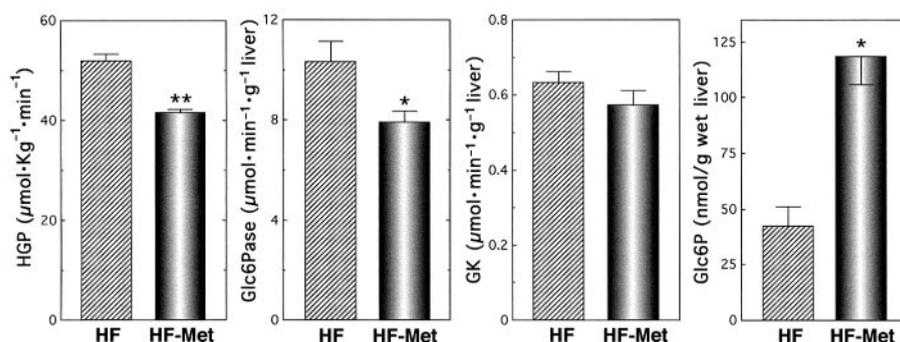The glycogen content was fivefold higher in the liver of

FIG. 1. Determination of basal HGP, Glc6Pase, and GK activities and Glc6P concentration in the liver of HF and HF-Met rats. HGP was assessed and calculated as described in RESEARCH DESIGN AND METHODS. Glc6Pase and GK activities and Glc6P concentrations were assessed in a liver lobe freeze-clamped in situ at the end of the experiment. The results are expressed as the means ± SE ($n = 5$). *$P < 0.05$ and **$P < 0.01$ vs. control.
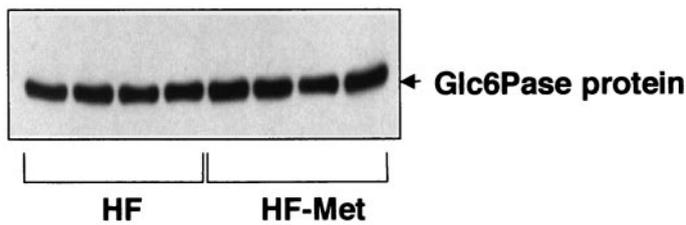
FIG. 2. Quantification of immunoreactive Glc6Pase protein in HF and HF-Met rats. Twenty-five μg protein was analyzed in each track. Procedures for Western blots were as described previously (7,21). The densitometric analysis did not reveal any significant difference between the groups.

HF-Met rats than in the liver of HF rats (19.6 ± 4.6 vs. 3.9 ± 2.4 mg/g wet tissue, respectively; $P < 0.01$) (Fig. 3). The glycogen content in control starch-fed rats (13.6 ± 4.2 mg/g) was higher than that in HF rats ($P < 0.05$) and was not different from that in HF-Met rats. In contrast, there was no difference in either the activity of GSa or GPa, irrespective of the diet given (Fig. 3).

There was a significant higher Glc6PDH activity in the liver of HF-Met rats as compared with HF rats (1.51 ± 0.1 vs. 1.06 ± 0.08 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet liver; $P < 0.01$). The liver Glc6PDH activity in the control group (1.75 ± 0.12 $\mu mol \cdot min^{-1} \cdot g^{-1}$) was not different from that in the HF-Met group and was significantly higher than that in the HF group ($P < 0.01$). The content in liver TG was not different in HF and HF-Met rats (16.4 ± 3.7 and 29 ± 7 mg/g liver, respectively; NS). In contrast, the liver TG content was lower in control starch-fed rats (4.4 ± 1.7 mg/g; $P < 0.05$ versus both HF groups).

Last, we determined the concentrations of intrahepatic lactate, the main precursor of liver gluconeogenesis. The latter tended to be lower in HF-Met rats with regard to HF rats (0.81 ± 0.06 vs. 1.11 ± 0.17 nmol/g wet liver, respectively, $n = 5$). The difference did not reach significance, however.

DISCUSSION

It has been strongly suggested that HF diets induce liver insulin resistance (6,23) and that the severity of insulin resistance is increasingly dependent on the duration of the HF feeding (24). However, proving insulin resistance of endogenous glucose production in HF-fed rats is not an easy task because of two major problems: *1*) because of low glucose content, HF diets generate lower plasma insulinemia as compared with starch diets (6,23); and *2*) because of very different substrate availabilities, HGP in HF-fed rats is unexpectedly lower than that in starch-fed rats under comparable postabsorptive conditions (6,23, present results). Liver insulin resistance in HF rats herein was clearly evidenced because HGP is shown to be much less efficiently inhibited by insulin in both groups of HF rats as compared with starch-fed control rats, despite higher final insulin levels (see RESULTS). It must be mentioned that we have checked in preliminary experiments that these final insulin levels allowed us to obtain a maximal suppression of HGP in the respective HF groups (not shown). It is also of note that rats fed the HF diet for 6 weeks are clearly hyperinsulinemic in the postabsorptive state ($\sim$280–300 pmol insulin/l) as compared with rats fed the same diet for only 3 weeks ($\sim$110–120 mol/l) (6).

In our previous study (6) with rats fed for 3 weeks the HF diet, which exhibited a moderate hepatic insulin resistance as compared with rats fed for 6 weeks, there was no effect of Met on basal HGP. The key result in the present study is that Met, incorporated in the HF diet at a dose close to the human therapeutic dose, significantly lowers HGP by 20%, whereas plasma glucagon, insulin, and glucose, the main regulatory factors of HGP (7,25,26), are similar in both the treated and untreated groups. This strongly suggests that rats fed an HF diet for 6 weeks constitute a suitable model to investigate the decreasing effect of Met on basal HGP, its major feature of action in diabetic humans (2–5). It should be mentioned that, because the disappearance rate of glucose is equal to the appearance rate (HGP) under our conditions of steady-state equilibrium determinations, the decreasing effect of Met on HGP might also be interpreted in terms of decreasing effect in basal glucose uptake. This suggests that the beneficial action of Met on the basal insulin sensitivity in the liver might counterbalance a worsening action on insulin sensitivity in the peripheral tissues. However, even if the difference is not significant ($P > 0.05$), the mean plasma insulin is 16% lower in HF-Met than in HF rats (Table 1). This may explain why the disappearance rate of glucose is decreased by 20% in HF-Met rats, suggesting that peripheral insulin sensitivity is not altered by Met.

Noteworthy is that the lowering in HGP in HF-Met rats may be ascribed to a major effect on Glc6Pase activity, which is decreased to a similar extent and restored to the level found in control rats. This decreasing effect is mediated via a suppression in the specific activity of the enzyme and not via a decrease in the amount of immunoreactive protein. One possible explanation might be that the Glc6Pase control mechanism, occurring in the presence of both glucose and insulin in normal rats (7), is impaired in insulin-resistant HF rats and restored by Met. Conversely, GK activity is low in HF rats as compared with control rats. This is in keeping with our previous results and with the state of hepatic insulin resistance in HF-fed rats (6). However, it seems that GK does not play a role in the Met effect on HGP because the GK activity is the same in both treated and untreated groups. In keeping with the occurrence of a high Glc6Pase flux, the Glc6P content is lowered in HF rats. The dramatic restoring action of Met on the Glc6P level in the HF-Met group is in strong
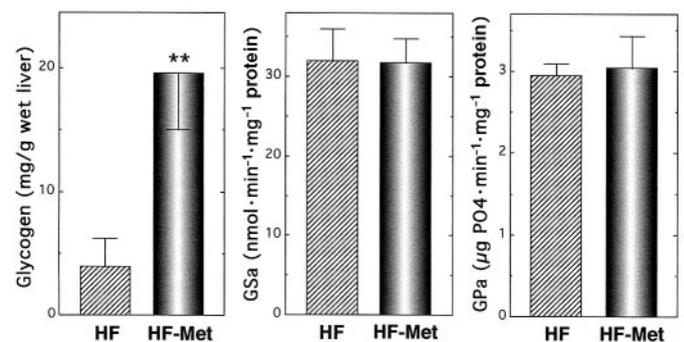

FIG. 3. Determination of the parameters of liver glycogen metabolism in HF and HF-Met rats. All three parameters were studied in the homogenates from freeze-clamped liver lobes at the end of the experiment (see Fig. 1). The results are expressed as means $\pm$ SE ($n = 5$). **$P < 0.01$ vs. control.

agreement with the inhibition of the substrate flux through Glc6Pase.

The liver glycogen content is very low in HF rats. It should be mentioned that a liver glycogen content lower than that of normal humans has been reported in patients with type 2 diabetes (27). Noteworthy is that glycogen stores are markedly restored to a level close to that in control rats by the Met treatment. Because neither GSa nor GPa has been altered by Met, the likely mechanism may involve either the increased availability in Glc6P ("push" effect) or, additionally, a putative activation of GS by Glc6P (28). Another possible fate for Glc6P could be to enter the pentose-phosphate pathway. This might result in the production of $NADPH+H^+$ and in the stimulation of lipogenesis. In this case, the biochemical mechanism should involve both the increase in the Glc6P concentration and the restoration of the enzyme controlling the first step, because Glc6PDH activity is the same in the HF-Met and control groups, whereas it is markedly lower in the HF group. The same phenomenon was very recently reported to occur under Met treatment in insulin-resistant fructose-fed rats (29). This might concur in the diversion of the Glc6P flux from Glc6Pase and hepatic output to the benefit of the pentose-phosphate pathway. This hypothesis could not be confirmed because TG stores were not significantly higher in HF-Met rats than in HF rats. A last possibility for Glc6P could be to increase the glycolytic flux, resulting in the intrahepatic production of lactate. However, the intrahepatic lactate concentration tends to be decreased rather than augmented in Met-treated rats with regard to their nontreated counterparts. In contrast, the plasma lactate concentration is slightly higher in HF-Met rats than in HF rats. This strongly suggests that, in addition to its downstream inhibitory action on glucose production at the level of Glc6Pase, Met has an upstream suppressive effect on the lactate uptake by the liver. This corroborates recent data obtained from perfused rat livers (30).

The decreasing effect of Met on HGP has been previously ascribed to the inhibition of either gluconeogenesis (2,3,30) or glycogenolysis (5). We emphasize that the major effect of Met on Glc6Pase activity reported herein strongly suggests that both pathways may be affected as well, because Glc6Pase is a common step for both gluconeogenesis and glycogenolysis. Noteworthy is that an increase in the Glc6Pase flux (31,32) and Glc6Pase V_{max} (33) has been strongly suggested to be involved in the increase in HGP in type 2 diabetes (see Ferrannini and Groop [34] as a review). In addition, the adenovirus-mediated overexpression of Glc6Pase in normal rats has been demonstrated to result in increased glucose production and decreased hepatic Glc6P concentration and glycogen content in cultured hepatocytes (35) and in rat liver in vivo (36). That the Met-induced suppression of Glc6Pase in HF rats may be causal with regard to the decrease in HGP and restoration of Glc6P concentration and glycogen content seems a highly relevant hypothesis.

In conclusion, our results strongly suggest that the decreasing action of Met on basal HGP in insulin-resistant HF-fed rats involves a major effect of inhibition of Glc6Pase activity. This may in turn allow a sparing effect on hepatic Glc6P and, consequently, on glycogen stores. In addition, an independent restoration of the pentose-phosphate pathway at the level of Glc6PDH activity and inhibition of the liver lactate uptake might concur in the Met action. Taken as a whole, the data presented herein suggest that rats on an HF diet for 6 weeks are very useful in the understanding of type 2 diabetes in humans and of the mechanisms of action of active drugs.

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